REMARKS

I. Status of Claims

Claims 1-16 are pending and stand rejected under 35 U.S.C. §103. The specific grounds for rejection, and applicants' response thereto, are set out in detail below.

II. Rejection Under 35 U.S.C. §103

Claims 2, 3 and 9-16 are rejected under §103 as obvious over Esmon and Kurosawa *eta al.* in further view of Hirsh *et al.* According to the examiner, Esmon discloses that hirudin, a specific thrombin inhibitor, blocked thrombin mediated increases in circulating EPCR, and that circulating EPCR levels are thus a surrogate for thrombin levels. Moreover, the examiner points to the following passage:

> This [monitoring of plasma EPCR] could prove useful in monitoring the progression of cardiovascular disease or the effectiveness of therapeutic interventions in these [human] patients.

Esmon at page 255. From this, it is concluded that the reference adequately discloses the use of sEPCR assays to monitor the effectiveness of anticoagulant therapies. Kurosawa *et al.* is cited as teaching the detection of sEPCR by ELISA of patient plasma. Hirsh is cited for teaching various anticoagulant therapies, and measuring the effectiveness of the anticoagulant therapy. Applicants traverse.

As discussed in the previous response, the cited passages from Esmon refer to the studies by Gu *et al.* (2000) in which endotoxin or thrombin treatment elevated soluble EPCR levels in a rat experimental model. These are ***inflammatory*** mediators in sepsis, and the effect of thrombin was blocked by hirudin, a specific thrombin inhibitor. By way of discussing their results, both Gu *et al.* and Esmon extrapolated these observations in a rat model to the human condition by

stating that monitoring plasma EPCR levels ***may*** indicate thrombin-mediated large vessel disease activity, and ***might*** prove useful to monitor disease progression or effectiveness of therapeutic interventions in these patients.

However, the study described in Gu *et al.* (2000) was designed to investigate the mechanism by which soluble EPCR is generated using an *in vivo* model. It was not designed to investigate how soluble EPCR levels change in patients as a result of anticoagulant therapy. Their data predicts that soluble EPCR levels may be linked to thrombin production in humans, but they do not test the prediction. They do not show data from patients or other humans in their study

The need for conducting studies in humans is at the crux of the rejection, namely, the lack of likelihood of success in practicing the claimed invention based only animal studies. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991). The rejection is based on the assertion that observations on sEPCR levels in a rat experimental model are predictive of levels in the human condition. The scientific literature contains examples in which this is true, and others in which this prediction is ***not*** true. Thus, there is an inherent caveat absolutely required for interpretation of data collected in an animal model, namely, that such data are clearly representative of the animal model, but may not be representative of similar response in humans (or other animal models for that matter).

The examiner dismissed applicants' previous arguments by concluding that the argument is simply "not persuasive." The examiner again pointed to the previously quoted portion of Esmon, and further noted that mutations in both human and murine thrombin genes can lead to thrombosis. However, the binding of thrombin to its receptor, which initiates signaling and the

production of sEPCR, is distinct from its clotting function, which is an acellular phenomenon. Thus, this observation is not particularly relevant.

Giudici *et al.* (1999) is cited in this same vein, as teaching that both human and mice can be protected from sepsis by antithrombin. In fact, Giudici *et al.* did ***not*** report that humans can be protected from sepsis by antitrhombin, with the results of the trial being very disappointing. All that this paper demonstrates is that antithrombin therapy ***may*** be effective in only a very small sub-population of patients with sepsis. This sub-population is very closely defined as patients with all of (a) sepsis, (b) septic shock, (c) requiring hemodynamic support and (d) antithrombin levels <70% of normal at the time of admission. This is ***not*** merely "patients with sepsis." Furthermore, there was ***no*** difference between placebo or antithrombin treatment on overall mortality beyond 30 days. From a clinical perspective, the patient sub-population affected was so narrowly defined as to be useless on a practical basis. As a result, antithrombin therapy is not recommended for use in sepsis.

Nonetheless, the examiner concludes that though "animal models are not ... exact replicas for human responses, but ... have and continue to serve as valuable models for many human diseases/conditions." Clearly, rodents provide valuable information regarding human disease states. That is not the issue here. The question is whether one can extrapolate from the animal studies of Esmon such that there would be ***reasonable*** predictability in a comparable human system. In order to more fully understand the issue, applicants wish to provide a more detailed discussion of the invention and it's development.

As discussed, the invention relates to measuring soluble EPCR in humans. The inventors are the first to show that soluble EPCR levels in are related to thrombin levels. The soluble EPCR seen in the human assay is released from the membrane-bound parent by

metalloproteinase activity. The metalloproteinase activity is up-regulated through a series of cellular signaling events arising from thrombin's interaction with cells that express EPCR. These receptors are called protease-activated receptors (PARs), of which several have now been identified.

The important point to be made about PARs is that humans utilize PAR-1 and PAR-4. Kahn *et al.* (1999; attached). In contrast, mice do not have PAR-1, and use PAR-3 and PAR-4 instead (Kahn *et al.*, 1998).[1] Thus, the inventors were prompted to perform the experiments described in the instant patent application because the they were aware that thrombin receptors differed between mice and humans (not to mention other species) and the signaling pathways are, therefore, different. See, for example, Connolly *et al.* (1994; attached). Thus, the results of Esmon, though interesting, did not provide *a priori* predictability with regard to sEPCR levels and thrombin activity in humans. This point was made in the previous response, and the examiner did not address this key issue. Thus, applicants submit that because mice and humans (not to mention other species) have different PAR receptors, there was no reason to believe that what Esmon showed was true for thrombin-thrombin receptor interactions in a mice would predictably translate to humans. This unrebutted fact is far more relevant than the examiner's citations.

Moreover, the examiner has improperly dismissed the impact of genetic variability on the results seen here. Murine populations used for studies like those described by Esmon *et al.* are inbred. While that may not have a significant impact in certain situations (for example, orthotopic cancer models), it most certainly has an impact when dealing with the complicated response seen with regard to sepsis and clotting. Even where positive results are seen in mice,

[1] With regard to PAR-2, the distinction between species is not clear, and PAR-2 is used by many enzymes other than thrombin.

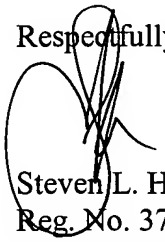
there is a need to confirm the results in human, and the literature is replete with examples where the murine results could ***not*** be confirmed in humans.

In sum, applicants submit that a fair review of the record indicates that the examiner arguments in favor of extrapolating from mice to humans in the context of thrombin and sEPCR are far less compelling than applicants' arguments to the contrary. On this basis, it is submitted that there was an insufficient likelihood of success in practicing the claimed invention, and therefore no *prima facie* case of obviousness exists. Reconsideration and withdrawal of the rejection based on the preceding discussion is respectfully requested.

III. Conclusion

In light of the foregoing, applicants submit that all claims are in condition for allowance, and an early notification to that effect is earnestly solicited. Should the examiner have any questions regarding this response, a telephone call to the undersigned is invited.

Respectfully submitted,



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Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin

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Because of the role of thrombin and platelets in myocardial infarction and other pathological processes, identifying and blocking the receptors by which thrombin activates platelets has been an important goal. Three protease-activated receptors (PARs) for thrombin — PAR1, PAR3, and PAR4 — are now known. PAR1 functions in human platelets, and the recent observation that a PAR4-activating peptide activates human platelets suggests that PAR4 also acts in these cells. Whether PAR1 and PAR4 account for activation of human platelets by thrombin, or whether PAR3 or still other receptors contribute, is unknown. We have examined the roles of PAR1, PAR3, and PAR4 in platelets. PAR1 and PAR4 mRNA and protein were detected in human platelets. Activation of either receptor was sufficient to trigger platelet secretion and aggregation. Inhibition of PAR1 alone by antagonist, blocking antibody, or desensitization blocked platelet activation by 1 nM thrombin but only modestly attenuated platelet activation by 30 nM thrombin. Inhibition of PAR4 alone using a blocking antibody had little effect at either thrombin concentration. Strikingly, simultaneous inhibition of both PAR1 and PAR4 virtually ablated platelet secretion and aggregation, even at 30 nM thrombin. These observations suggest that PAR1 and PAR4 account for most, if not all, thrombin signaling in platelets and that antagonists that block these receptors might be useful antithrombotic agents.

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Introduction

Platelet activation is critical for normal hemostasis, and platelet-dependent arterial thrombosis underlies most myocardial infarctions. Thrombin is the most potent activator of platelets (1, 2). Characterization of the receptors that mediate thrombin's actions on platelets is therefore necessary for understanding hemostasis and thrombosis. Moreover, such receptors are potential targets for novel antiplatelet therapies.

Thrombin signaling is mediated at least in part by a family of G protein–coupled protease-activated receptors (PARs), for which PAR1 is the prototype (3, 4). PAR1 is activated when thrombin cleaves its NH_2-terminal exodomain to unmask a new receptor NH_2-terminus (3). This new NH_2-terminus then serves as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect transmembrane signaling (3, 5, 6). The synthetic peptide SFLLRN, which mimics the first six amino acids of the new NH_2-terminus unmasked by receptor cleavage, functions as a PAR1 agonist and activates the receptor independent of proteolysis (3, 7, 8). Such peptides have been used as pharmacological probes of PAR function in various cell types.

Our understanding of the role of PARs in platelet activation is evolving rapidly. PAR1 mRNA and protein were detected in human platelets (3, 9–11), SFLLRN activated human platelets (3, 7, 8), and PAR1-blocking antibodies inhibited human platelet activation by low, but not high, concentrations of thrombin (9, 10). These data suggested a role for PAR1 in activation of human platelets by thrombin but left open the possibility that other receptors might contribute.

Curiously, PAR1 appears to play no role in mouse platelets. PAR1-activating peptides did not activate rodent platelets (12–14), and platelets from PAR1-deficient mice responded like wild-type platelets to thrombin (14). This observation prompted a search for additional thrombin receptors and led to the identification of PAR3 (15). PAR3 is activated by thrombin and is expressed in mouse platelets. PAR3-blocking antibodies inhibited mouse platelet activation by low, but not high, concentrations of thrombin (16), and knockout of PAR3 abolished mouse platelet responses to low, but not high, concentrations of thrombin (17). These results established that PAR3 is necessary for normal thrombin signaling in mouse platelets but also pointed to the existence of another mouse platelet thrombin receptor. Such a receptor, PAR4, was recently identified (17, 18). PAR4 appears to function in both mouse and human platelets (17). Thus, available data suggest a testable working model in which PAR3 and PAR4 mediate thrombin activation

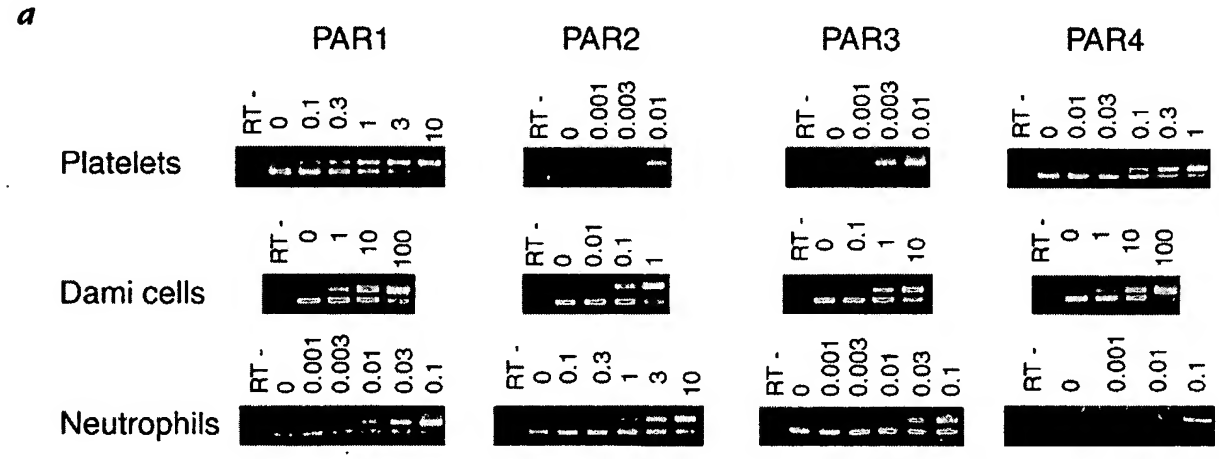
of mouse platelets and PAR1 and PAR4 mediate activation of human platelets. The role of PAR3, if any, in human platelets has not been determined. More broadly, the relative roles of PAR1, PAR3, and PAR4, and whether still other receptors also contribute to platelet activation by thrombin, are unknown.

To determine the roles of PAR1, PAR3, and PAR4 in activation of human platelets by thrombin, we examined expression of receptor mRNA and protein in platelets and probed receptor function with specific peptide agonists. We also examined the effect of receptor desensitization, receptor-blocking antibodies, and a PAR1 antagonist, used alone and in combination, on platelet activation. Our results suggest that PAR1 and PAR4 together account for most, if not all, thrombin signaling in human platelets. PAR3, while important for thrombin signaling in mouse platelets, appears to have little or no role in human platelets. These results are potentially important for the development of antiplatelet therapies.

Methods

Measurement of PAR mRNA levels by competitive reverse transcription-PCR. Dami cells (19) were grown in suspension in RPMI with 10% FBS. Platelet preparations (17) contained <0.1% leukocytes as assessed by light microscopic analysis. A discontinuous Percoll gradient was used to separate monocytes and lymphocytes from neutrophils, according to the manufacturer's instructions (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). The monocyte/lymphocyte preparations contained <0.1% neutrophils, and the neutrophil preparations contained <0.1% monocytes or lymphocytes. Total RNA was prepared from all cells using Trizol Reagent (GIBCO BRL, Grand Island, New York, USA), treated with DNase (Boehringer Mannheim, Indianapolis, Indiana, USA), and quantified by OD 260.

Competitor RNA templates for each receptor were created by mutating the respective cDNA to ablate an endogenous restriction endonuclease site (see below), and competitor RNAs were generated by *in vitro* transcription. Reverse transcription (RT) reactions were performed using 200 ng of total cellular RNA mixed with varying amounts of competitor RNA in a 10-µl reaction volume using a commercial kit (GIBCO BRL) and receptor specific primers (see below). RT product (2 µl) was amplified by PCR in a 50-µl volume containing a final concentration of 2 µM primers (see below) and 5 U *Taq* polymerase (GIBCO BRL). Reaction conditions were 94°C for 4 min, 72°C for 1 min with addition of *Taq*, then 94°C for 45 s, 55°C for 1 min, 72°C for 1 min for 30–36 cycles (see below), and then 72°C for 8 min. Cycle numbers and concentration ranges for competitor RNAs were chosen for each sample in preliminary experiments. The number of cycles chosen for measurement of PAR1, PAR2, PAR3, and PAR4 mRNA levels, respectively, in RNA from the various sources follows.



b

	PAR1	PAR2	PAR3	PAR4
	(attomoles/200 ng total RNA)			
Platelets (1)	1	0.001	< 0.001	0.3
(2)	0.3 - 1	< 0.001	< 0.001	0.1
Dami cells	10	0.1	1 - 10	10
Neutrophils	0.01	3	0.03 - 0.1	< 0.01
Monocytes + Lymphocytes	1	0.3	0.03 - 0.1	0.3

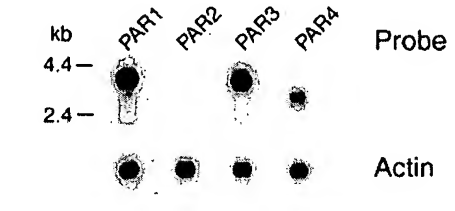


Figure 1

Expression of mRNAs encoding PAR1, PAR2, PAR3, and PAR4 in platelets, Dami cells, and neutrophils. (*a*) Competitive RT-PCR of total RNA from platelets, Dami cells, and neutrophils. Total cellular RNA (200 ng) mixed with the indicated quantity of competitor RNA (measured in attomoles) was reverse-transcribed and amplified. Products were digested with a restriction endonuclease to distinguish the products of competitor RNA (*uncleavable upper band*) vs. native cellular mRNA (*lower band*). RT indicates mock RT-PCR of total cellular RNA and the highest amount of competitor RNA with no reverse transcriptase added. Each sample was analyzed at least twice. Note that the single band seen in the platelet PAR3 RT-PCR is due to amplification of competitor RNA. (*b*) Quantitation of PAR mRNAs in platelet, Dami cell, neutrophil, and monocyte/lymphocyte preparations. Results indicate the range of values obtained from at least two experiments like that shown in *a*. 1 amol/200 ng corresponds to an mRNA relative abundance of roughly 1:3,000. The expression of PAR mRNA in the platelets of two unrelated individuals is shown. (*c*) Northern blot analysis of PAR gene expression in Dami cells. Blots were hybridized separately with coding region probes for PAR1, PAR2, PAR3, or PAR4, as well as with probe for β-actin mRNA as a control for lane loading. Note concordance with PCR data in *b*. *PAR*, protease-activated receptor; *RT*, reverse transcription.

Platelets: 31, 36, 36, 36; neutrophils: 36, 27, 31, 36; monocytes/lymphocytes: 31, 31, 33, 36; and Dami cells: 30, 32, 32, 33.

Primers used for RT and PCR of each receptor and the restriction endonuclease used to digest each PCR product. Nucleotide numbering is such that 1 equals the A of the start ATG.

PAR1, GenBank accession no. M62424: Primer for RT: TAG ACG TAC CTC TGG CAC TC (1148-1129). Sense-strand primer for PCR: CAG TTT GGG TCT GAA TTG TGT CG. Antisense primer for PCR: TGC ACG AGC TTA TGC TGC TGA C. Resulting PCR product: 505-1096.Mutated site: *AgeI* at position 596.

PAR2, GenBank accession no. U34038: Primer for RT: CTG CTC AGG CAA AAC ATC (699-682). Sense-strand primer for PCR: TGG ATG AGT TTT CTG CAT CTG TCC. Antisense primer for PCR: CGT GAT GTT CAG GGC AGG AAT G. Resulting PCR product: 182-672. Mutated site: *SfiI* at position 342.

PAR3 GenBank accession no. U92972: Primer for RT: TGA TGT CTG GCT GAA CAA G (727-709). Sense-strand primer for PCR: TCC CCT TTT CTG CCT TGG AAG. Antisense primer for PCR: AAA CTG TTG CCC ACA CCA GTC CAC. Resulting PCR product: 152-664. Mutated site: *NcoI* at position 251.

PAR4, GenBank accession no. AF080214: Primer for RT: TGA GTA GCT GGG ATT ACA G (1519-1501). Sense-strand primer for PCR: AAC CTC TAT GGT GCC TAC GTG C. Antisense primer for PCR: CCA AGC CCA GCT AAT TTT TG. Resulting PCR product: 949-1490. Mutated site: *Bam*HI at position 1005.

After PCR amplification, 10 μl of reaction product was digested overnight with the appropriate restriction endonuclease and analyzed by 1.5% agarose gel electrophoresis. The products of reactions that included only native mRNA were completely cleaved by the appropriate restriction endonuclease, while the products of reactions that included only competitor RNA remained undigested (Fig. 1 and data not shown). By adding varying amounts of competitor RNA to total cellular RNA before RT-PCR and determining the competitor RNA concentration at which the intensity of the competitor RNA-derived product (uncleaved band) matched that of the endogenous mRNA-derived product (cleaved band), the quantity of each PAR mRNA in the original sample was estimated.

Northern blot analysis. Poly(A)$^+$ RNA (2 μg) derived from Dami cells was electrophoresed, transferred to nitrocellulose membranes, and hybridized under high-stringency conditions. PAR1 mRNA was detected with a 400-bp *PstI*/*PvuII* cDNA probe; PAR2 mRNA was detected with a 260-bp *SfiI*/*Bst*EII cDNA probe; PAR3 mRNA was detected with a 610-bp *KpnI*/*NsiI* cDNA probe; PAR4 mRNA was detected using a 450-bp *SacI*/*PstI* cDNA.

Generation and characterization of PAR polyclonal antibodies. The synthetic peptides GGDDSTPSILPAPRGYPGQVC (PAR4 amino acids 34–55), AKPTLPIKTFRGAPPNSFEEFPFSALEGC (PAR3 amino acids 31–58 plus carboxyl glycine-cysteine) and NATLDPRSFLLRNPNDKYEPFWEDEEGC (PAR1 amino acids 35–61 plus carboxyl glycine-cysteine) were conjugated to keyhole limpet hemocyanin and used to generate polyclonal antisera in rabbits. IgG was purified by protein-A affinity chromatography to generate the PAR4, PAR3, and PAR1 IgG preparations used in this study. Binding of these IgGs and PAR4 preimmune IgG to each receptor was tested on COS cells transiently expressing FLAG epitope-tagged receptors using an enzyme-linked immunosorbent assay (ELISA) (16, 20). cDNA for an epitope-tagged human PAR4 analogous to FLAG epitope-tagged PAR1 was constructed as described previously (15, 20) such that the FLAG epitope was fused to amino acid 22 in PAR4 to yield the following sequence: ... DYKDDDDVE/TPSVYD ... (where the slash indicates the junction with PAR4 sequence).

Flow cytometry. Washed platelets (17) and Dami cells were fixed with paraformaldehyde, incubated with PAR1 or PAR3 IgG at 10 μg/ml or PAR4 IgG at 100 μg/ml, washed, incubated with FITC-conjugated goat anti-rabbit IgG, washed, and then analyzed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Some fixed platelet samples were exposed to 30 nM thrombin at 37°C before incubation with primary antibody.

Functional studies in Xenopus oocytes. FLAG epitope-tagged PAR4 cDNA was subcloned into pFROG (3) to permit *in vitro* transcription of PAR4 cRNA. Signaling studies were performed in *Xenopus* oocytes microinjected with 2.0 ng of PAR4 cRNA or 25 ng of PAR1 cRNA per oocyte (3, 21).

Platelet aggregation and secretion. Aggregation and secretion were measured using washed human platelets (17). For desensitization studies, platelets resuspended from the first platelet pellet were incubated with SFLLRN (100 μM) or GYPGKF (500 μM) in the presence of prostaglandin E_1 (PGE_1) at room temperature for 30 min without stirring then washed by centrifugation (17).

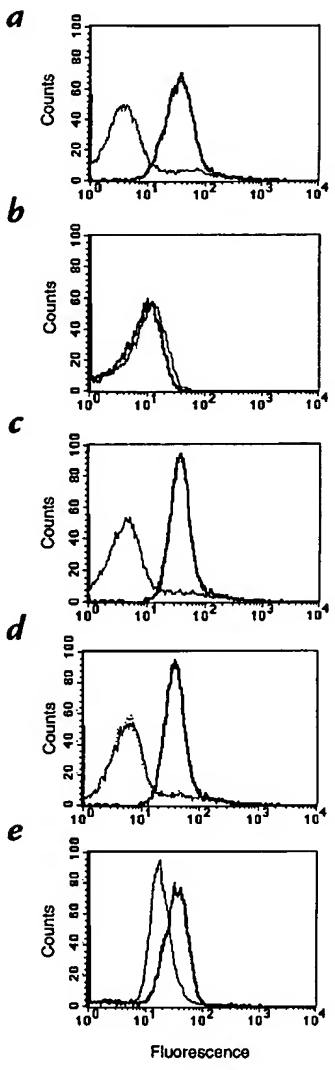


Figure 2
Flow cytometric analysis of platelets for surface expression of PAR1, PAR3, and PAR4. Fixed platelets were incubated with preimmune IgG (*narrow lines*) or PAR1 IgG (*a*), PAR3 IgG (*b*), or PAR4 IgG (*c*) (*wide lines*) and then analyzed as described in Methods. (*d*) Platelets were incubated with PAR4 IgG in the absence (*wide line*) or presence (*thin line*) of the peptide antigen (1 μM) used to generate the PAR4 antiserum, or after treatment with 20 nM thrombin for 10 min at 37°C (*dotted line*). Each curve represents an analysis of 10,000 events. This experiment was repeated twice with separate donors with equivalent results. (*e*) Flow cytometric analysis of Dami cells as a positive control for detection of PAR3. Fixed Dami cells were incubated with preimmune IgG (*narrow line*) or PAR3 IgG (*wide line*) and then analyzed as above. Dami cells were also positive for PAR1 and PAR4 (not shown).

For functional studies with PAR1 or PAR4 antibody, washed platelets were incubated with antibody or preimmune IgG for 60 min before measurement of secretion and aggregation. PAR1 antagonist was added to stirring platelets 1–2 min before the addition of thrombin or other agonists.

Measurement of receptor cleavage by thrombin. Rat-1 fibroblasts were stably transfected with FLAG epitope-tagged PAR1 and PAR4 expression vectors (22), and cleavage of surface receptors was followed as described previously (20).

Measurement of PAR1 and PAR4 signaling. A *Par1*$^{-/-}$ mouse lung fibroblast cell line that showed no thrombin signaling (14, 23) was used to generate stable cell lines expressing FLAG epitope-tagged PAR1 and PAR4. Increases in cytoplasmic calcium in response to thrombin were measured using the calcium-sensitive dye Fura-2 as described previously (14).

Results

Expression of PAR mRNAs in platelets and other blood cells. To validate an RT-PCR assay for PAR mRNAs, Dami cells, a human cell line that expresses some megakaryocyte markers (19), were analyzed. Competitive RT-PCR of Dami cell RNA (Fig. 1, *a* and *b*) revealed the presence of PAR1, PAR3, and PAR4 mRNA; PAR2 mRNA was also detected but at only 1% the level of PAR1 mRNA. Northern analysis was positive for PAR1, PAR3, and PAR4 but not PAR2 (Fig. 1*c*). At the protein level, PAR1, PAR3, and PAR4 were detected on the surface of Dami cells by flow cytometry (Fig. 2, and data not shown). Thus, results from RT-PCR were generally concordant with Northern and protein analysis.

Competitive RT-PCR of platelet RNA revealed PAR1 mRNA to be present at approximately 1 amol/200 ng total RNA. Assuming mRNA is 1% of total platelet RNA and an average mRNA size of 2 kb, PAR1 mRNA represents 1 in 3,000 platelet mRNAs. PAR4 mRNA was also detected at 10%–30% of PAR1 mRNA levels. By contrast, PAR3 mRNA was undetectable. PAR3 competitor RNA added to platelet RNA was detected at 0.001 amol/200 ng total RNA, suggesting that PAR3 mRNA was at least 1,000-fold less abundant than PAR1 mRNA in these samples. PAR2 mRNA was not detected in platelet RNA from one individual (no. 2), and only 0.001 amol/200ng was detected in the other (no. 1). The latter measurement may be due to trace contamination of the platelet preparation by neutrophils (see below), consistent with the observation that the specific PAR2 agonist peptide SLIGKV is unable to activate human platelets (data not shown).

The pattern of PAR mRNA expression in neutrophils and mononuclear cells was distinct from that seen in platelets, suggesting that contamination of platelet preparations by leukocytes did not significantly influence the PAR expression pattern detected in platelets. In particular, while virtually absent from platelets, substantial PAR2 mRNA was detected in both neutrophils and mononuclear cells. The relatively high PAR2 mRNA level in neutrophils is consistent with previous studies demonstrating neutrophil responses to PAR2-activating peptide (24). In contrast to platelets, PAR3 mRNA was consistently detected at low levels in mononuclear cells. PAR4 mRNA was also found in mononuclear cell preparations but not in neutrophils. These results demonstrate the presence of mRNA encoding PAR1 and PAR4, but not PAR2 or PAR3, in human platelets.

Expression of PAR proteins on the surface of human platelets. IgG was purified from rabbit antisera raised to peptides representing the NH_2-terminal exodomains of PAR1, PAR3, or PAR4. To assess ability to recognize native PARs and cross-reactivity, antibody binding to the surface of receptor-expressing COS cells was measured. Each IgG preparation bound to the surface of cells expressing the appropriate receptor without significant cross-reactivity (data not shown).

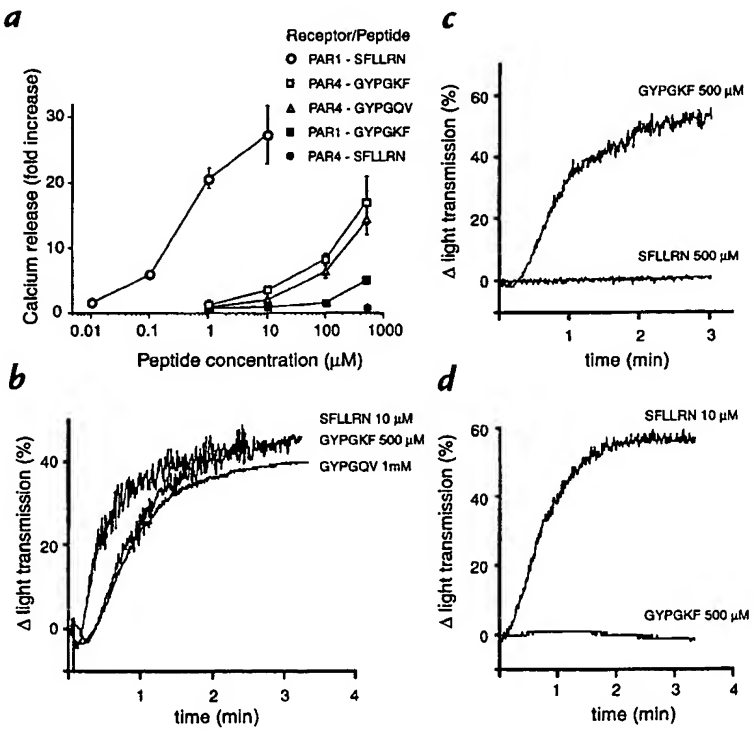
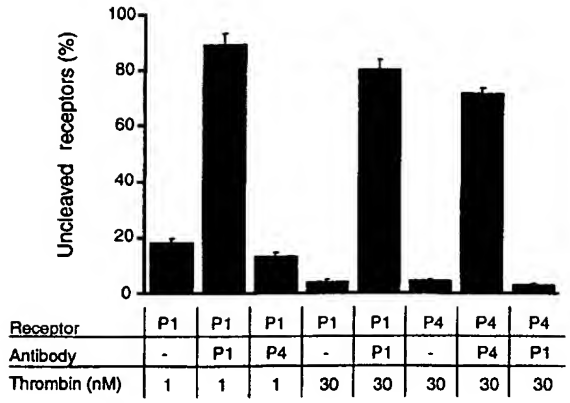


Figure 3
Effects of PAR1- and PAR4-activating peptides. (*a*) Specificity and potency. Peptide-triggered ^{45}Ca release was measured in *Xenopus* oocytes expressing human PAR1 and human PAR4 tagged at their NH_2-termini with a FLAG epitope. Data are mean ± SEM ($n = 3$) and are expressed as fold increase over baseline for each receptor. Surface expression of PAR1 measured with anti-FLAG monoclonal antibody was 1.3 times that of PAR4. This experiment was replicated twice. (*b–d*) Activation of human platelets with the PAR1-activating peptide SFLLRN and the PAR4-activating peptides GYPGKF and GYPGQV. (*b*) Platelets were exposed to either SFLLRN (10 μM) or GYPGKF (500 μM) or GYPGQV (1 mM) at time 0, and aggregation was measured as change in light transmission. (*c*) SFLLRN-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) at time 0, and aggregation was measured as change in light transmission. (*d*) GYPGKF-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) at time 0, and aggregation was measured as change in light transmission. The experiments in *b*, *c*, and *d* were repeated three times.

Figure 4
Inhibition of thrombin cleavage of receptor NH_2-terminus by anti-PAR1 and anti-PAR4 antibodies. Rat-1 cells expressing PAR1 and PAR4 bearing the FLAG epitope at their extreme NH_2-termini were fixed and then incubated with PAR1 IgG (*P1*; 100 μg/ml), PAR4 IgG (*P4*; 1 mg/ml), or buffer alone for 60 min before exposure to either 1 or 30 nM thrombin for 10 min at 37°C. Receptor cleavage was measured as loss of binding sites for M1 monoclonal antibody to the FLAG epitope, which was NH_2-terminal to the thrombin cleavage site in both receptors, so as to be lost from the cells upon receptor cleavage. Data (mean ± SEM; $n = 3$) are expressed as percent of control cells exposed to buffer alone. This experiment was repeated twice.



These IgG preparations were then used for flow cytometric analysis of human platelets (Fig. 2). Significant surface binding was detected with PAR1 IgG vs. preimmune IgG (Fig. 2*a*), consistent with previous studies (9, 11, 25, 26). A similar increase in platelet surface binding was detected with PAR4 IgG vs. PAR4 preimmune IgG (Fig. 2*c*). Preincubation of PAR4 IgG with the peptide antigen to which it was raised abolished this increase (Fig. 2*d*). Moreover, the epitope to which the PAR4 antiserum was raised spans PAR4's thrombin cleavage site, and treatment of platelets with thrombin indeed abolished PAR4 IgG binding (Fig. 2*d*). These data strongly suggest that PAR1 and PAR4 are expressed on the surface of human platelets.

PAR3 immune IgG showed no specific binding to human platelets (Fig. 2*b*). To determine whether this antibody preparation could detect PAR3 expressed at "natural" levels, this experiment was repeated with Dami cells (Fig. 2*e*, and data not shown), which had been shown by Northern blot to express PAR3 mRNA (Fig. 1). A significant increase in antibody binding was seen with PAR3 antibody vs. nonimmune IgG, consistent with RT-PCR and Northern blot analysis (Fig. 1). This suggests that the absence of detectable PAR3 protein on the surface of human platelets is not due to insensitivity of the assay. Taken together, these data confirm the presence of PAR1 and PAR4, but not PAR3, on the surface of human platelets.

Activation of human platelets by PAR1- and PAR4-activating peptides. Synthetic peptides that mimic the tethered ligands of PAR1 and PAR2 function as agonists for their respective receptors (3, 7, 8) and have been used as pharmacological tools to probe the function of these receptors in various cell types. Unfortunately, the cognate peptide for PAR3 appears to be insufficiently avid to function as a free ligand (15). We and others (17, 18) recently showed that a peptide mimicking the tethered ligand for PAR4 can function as an agonist for that receptor, albeit at a concentration higher than that seen with the PAR1 and PAR2 peptides and their cognate receptors. To determine the specificity of the PAR1 and PAR4 tethered ligand peptides, we first assessed their ability to trigger calcium mobilization in *Xenopus* oocytes heterologously expressing PAR1 and PAR4, our most sensitive assay system (Fig. 3*a*). No responses were detected in oocytes expressing neither receptor (not shown). Both the human PAR4 peptide GYPGQV and the mouse PAR4 peptide GYPGKF activated oocytes expressing human PAR4, but with an EC_{50} roughly two orders of magnitude higher than that of SFLLRN for PAR1 activation (Fig. 3*a* and ref. 17). SFLLRN showed no activity at PAR4. At 500 μM, the PAR4 peptide GYPGKF did show minimal activity at PAR1. However, because PAR1 is overexpressed such that the sensitivity for detection of PAR1 activation in the oocyte assay is 10- to 100-fold greater than in platelets, it is likely that PAR1 activation at 500 μM GYPGKF is unimportant in the platelet studies described below.

The PAR1 peptide SFLLRN and the PAR4 peptides GYPGKF and GYPGQV all activated human platelets (Fig. 3*b*). The PAR4 peptides were considerably less potent than the PAR1 peptide for activating human platelets; GYPGKF was slightly more potent than GYPGQV (Fig. 3*b*, and data not shown). The potencies of these peptides for platelet activation thus correlated with their relative potencies for activation of their respective receptors in the oocyte system (Fig. 3).

Incubation of PGE_1-treated platelets with SFLLRN rendered them refractory to subsequent stimulation by SFLLRN but did not affect responsiveness to GYPGKF (Fig. 3*c*). Conversely, incubation with GYPGKF rendered platelets refractory to subsequent stimulation by GYPGKF but did not affect responsiveness to SFLLRN (Fig. 3*d*). These results suggest that activation of either PAR1 or PAR4 with their cognate peptide agonists is sufficient to activate human platelets. Taken together, the results presented above show that PAR1 and PAR4 function in human platelets.

PAR1 and PAR4 antibodies inhibit thrombin cleavage of their respective receptors. To determine the necessary roles of PAR1 and PAR4 in platelet activation by thrombin, we developed blocking antibodies. The previously described PAR1 antibody raised against PAR1's hirudin-like domain (9) is predicted to inhibit thrombin cleavage of PAR1's NH_2-terminal exodomain by disrupting binding to thrombin's anion-binding exosite. Because no analogous hirudin-like domain was apparent in the sequence of PAR4's NH_2-terminal exodomain, antiserum was raised to a peptide that represented sequence spanning PAR4's thrombin cleavage site. This antiserum specifically recognized PAR4 (Fig. 2, and data not shown). To test the ability of the PAR1 and PAR4 antibodies to block cleavage of PAR1 and PAR4, Rat-1 fibroblasts expressing FLAG epitope-tagged PAR1 and PAR4 were preincubated with antibody. Receptor cleavage was then measured as loss of FLAG epitope from the cell surface upon expo-

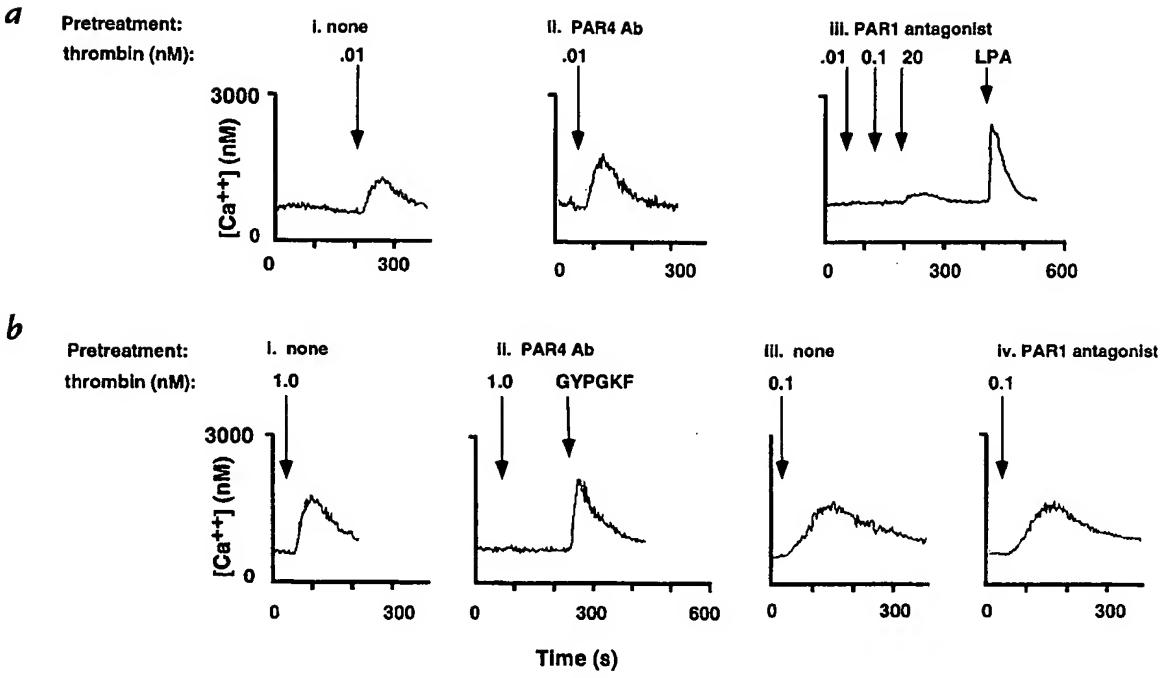


Figure 5

Inhibition of PAR1 and PAR4 signaling by PAR1- and PAR4-blocking antibodies and PAR1 antagonist. Fibroblast cell lines in which thrombin signaling was mediated solely by PAR1 (*a*) or by PAR4 (*b*) were incubated with buffer alone (*none*), PAR4 IgG (*PAR4 Ab*; 1 mg/ml), or the PAR1 antagonist BMS200261 (100 μM) for 30 min at 37°C. Cells were then exposed to thrombin (0.01, 0.1, 1.0, or 20 nM as indicated), GYPGKF (500 μM), or lysophosphatidic acid (*LPA*; 5 μM). Receptor-triggered increases in cytoplasmic calcium were measured fluorometrically using the calcium sensitive dye Fura-2. This experiment was repeated three times with similar results. *Ab*, antibody.

sure to thrombin (20) (Fig. 4). PAR1 cleavage was markedly inhibited by PAR1 antibody but not by PAR4 antibody. Conversely, PAR4 cleavage was markedly inhibited by PAR4 antibody, but not by PAR1 antibody. These data predicted that the PAR1 and PAR4 antibodies should selectively attenuate thrombin signaling via PAR1 and PAR4, respectively.

Inhibition of thrombin signaling by PAR1 and PAR4 antibodies, and by a PAR1 antagonist. A fibroblast cell line derived from PAR1-deficient mice (23) was used to generate lines stably expressing human PAR1 and PAR4. Because no thrombin responses were detectable in untransfected PAR1-deficient fibroblasts, signaling in the transfected cells could be attributed to the transfected receptor. In the PAR1-expressing cell line, increases in cytoplasmic calcium were reliably elicited by thrombin at concentrations as low as 10 pM (Fig. 5*a*). PAR4 IgG had no inhibitory effect, even on these threshold responses (Fig. 5). As demonstrated previously (9), PAR1 IgG markedly attenuated such signaling, and nonimmune antibody was without effect (data not shown).

The PAR1 antagonist BMS200261 (27) attenuated PAR1 signaling even at high thrombin concentrations (Fig. 5). Responsiveness to lysophosphatidic acid was unaffected by the antagonist, as was PAR4 signaling (Fig. 5, and data not shown), suggesting that the inhibitory effect of BMS200261 was specific.

In the PAR4-expressing cell line, increases in cytoplasmic calcium were reliably triggered at 1 nM thrombin (Fig. 5*b*). PAR4 IgG blocked such responses but had no effect on responses to GYPGKF, consistent with the antibody's acting by preventing receptor cleavage by thrombin. PAR4 preimmune IgG, PAR1 IgG, and PAR1 antagonist (100 μM) failed to inhibit PAR4 signaling even at threshold thrombin concentrations (Fig. 5*b*, and data not shown). Taken together, these results established specific tools for blocking PAR1 or PAR4. PAR1 and PAR4 could each be blocked with a specific IgG. PAR1 could also be specifically blocked with BMS200261 or by homologous desensitization with SFLLRN. This presented an opportunity to assess the roles of PAR1 and PAR4 in platelet activation by thrombin.

Inhibition of thrombin-induced platelet aggregation by blocking PAR1 and PAR4 signaling. The contribution of PAR1 and PAR4 signaling to thrombin activation of human platelets was tested using the strategies outlined above. By itself, PAR4 IgG had no effect on platelet aggregation, even at low (1 nM) thrombin (Fig. 6). By contrast, PAR1 IgG or BMS200261 markedly inhibited platelet aggregation in response to 1nM thrombin, as did prior desensitization of platelets with the PAR1 agonist SFLLRN (Fig. 6). None of these maneuvers inhibited platelet aggregation in response to GYPGKF or submaximal concentrations of adenosine diphosphate (ADP) (Fig. 6 and data not shown). These data suggest that PAR1 is the major mediator of platelet activation at low concentrations of thrombin, consistent with previous studies (9, 25).

In contrast to the case at 1 nM thrombin, at 30 nM thrombin, inhibition of PAR1 signaling by either PAR1 IgG, antagonist, or SFLLRN desensitization was largely ineffective, only slowing aggregation slightly, such that shape change became detectable (see 0- to 30-second portions of

the aggregation curves in Fig. 6, *b–d*). Inhibition of PAR4 signaling with PAR4 IgG was similarly ineffective (Fig. 6*b*).

Strikingly, when signaling via PAR1 and PAR4 was blocked simultaneously, aggregation in response to even high concentrations of thrombin was virtually abolished (Fig. 6). Such synergy was seen regardless of the means by which PAR1 was blocked (desensitization, PAR1 IgG, or antagonist) (Fig. 6). PAR4 preimmune IgG had no effect in such experiments (data not shown), and platelet activation by ADP and by GYPGKF, which bypasses the effect of the PAR4 antibody, were not inhibited (Fig. 6, and data not shown). These data strongly suggest that both PAR1 and PAR4 contribute to platelet activation at high (30 nM) concentrations of thrombin and that inhibition of both receptors is required to ablate thrombin-triggered platelet aggregation.

Inhibition of thrombin-induced platelet secretion by blocking PAR1 and PAR4 signaling. A more quantitative measure of platelet activation is the amount and rate of adenosine triphosphate (ATP) release due to the secretion of platelet granule contents. We therefore measured the effect of BMS200261, PAR4 IgG, or both, on peak ATP release and the time to half-maximal ATP release in response to 30 nM thrombin (Fig. 7). PAR1 antagonism with BSM200261 decreased maximal ATP secretion to approximately one-third of control levels and prolonged the time to half-maximal secretion by approximately threefold. PAR1 IgG alone had a similar effect (not shown). PAR4 antibody alone had no effect on the tempo of ATP secretion and only a small effect, if any, on peak response. Strikingly, the combination of BMS200261 and PAR4 IgG ablated ATP secretion in response to 30 nM thrombin. Even after 10 minutes, no secretion was detected. Similar data were obtained when PAR1 IgG was combined with PAR4 IgG. These maneuvers did not block secretion in response to GYPGKF (Fig. 7), and preimmune and nonimmune IgG were without effect (data not shown). These results support the hypothesis that PAR1 and PAR4 account for platelet secretion and aggregation in response to thrombin at concentrations as high as 30 nM. They also suggest that PAR1 is necessary for rapid platelet activation by thrombin even at high thrombin concentrations.

Discussion

In this study, we addressed the roles of PAR1, PAR3, and PAR4 in activation of human platelets by thrombin. We showed that PAR1 and PAR4 are functionally expressed in human platelets and that these receptors account for most if not all thrombin signaling in these cells. We further demonstrate that PAR1 mediates platelet responses at low concentrations of thrombin and is necessary for the most rapid and robust platelet responses, even at high concentrations of thrombin, consistent with previous studies (9, 10). In contrast to PAR1, PAR4 mediates platelet activation only at high thrombin concentrations and PAR4 signaling appears unnecessary for platelet activation when PAR1 function is intact.

The observation that specific inhibition of PAR1 and PAR4 ablate thrombin signaling in human platelets suggests thrombin binding to GPIbα (28) is not sufficient to trigger platelet activation; whether such binding plays any adjunctive role is not known. Our results also sug-

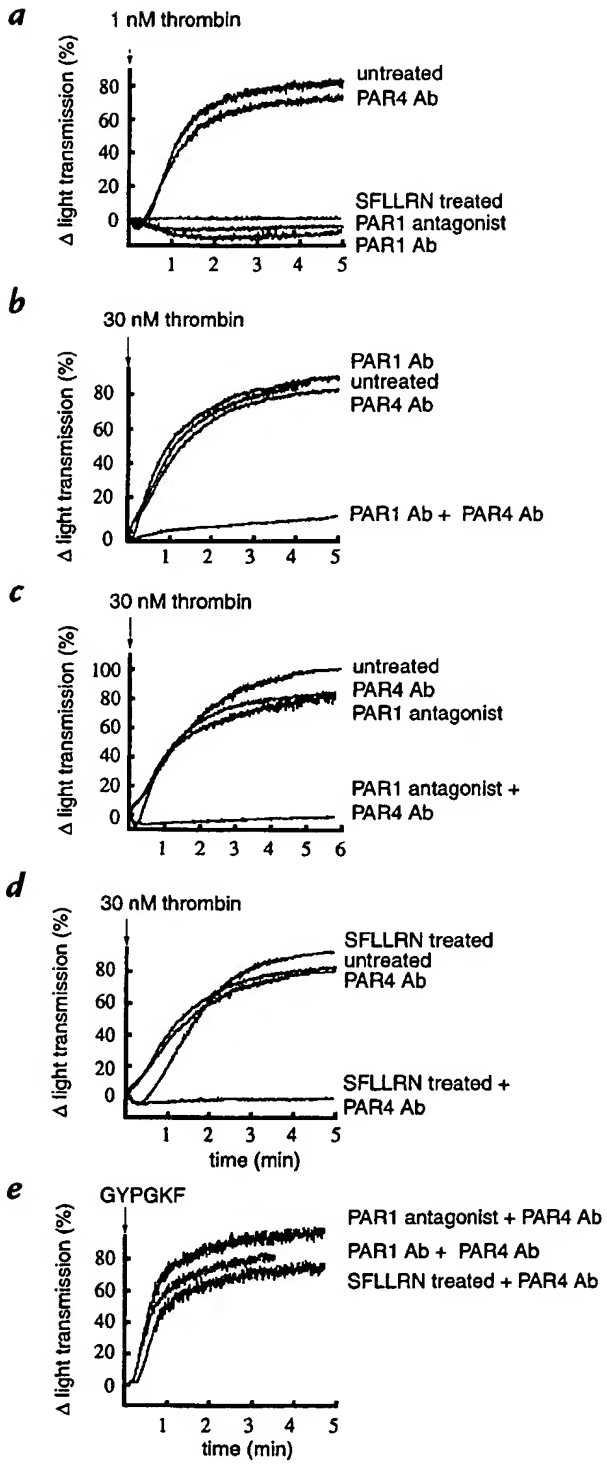


Figure 6

The effects of inhibition of PAR1 and/or PAR4 on aggregation of human platelets in response to low (1 nM) and high (30 nM) concentrations of thrombin. Platelets were pretreated with buffer alone, PAR1 IgG (10 μg/ml), PAR4 IgG (1 mg/ml), or PAR1 antagonist (100 μM), or were desensitized to SFLLRN as indicated and then exposed to 1 nM thrombin (*a*), 30 nM thrombin (*b–d*), or 500 μM GYPGKF (*e*) at time 0. Aggregation was measured as increase in light transmission. Preimmune or nonimmune IgG were without effect (not shown). This experiment was performed using triplicate samples twice (*a*, *c*, *e*) or four times (*b*, *d*). Representative tracings are shown.

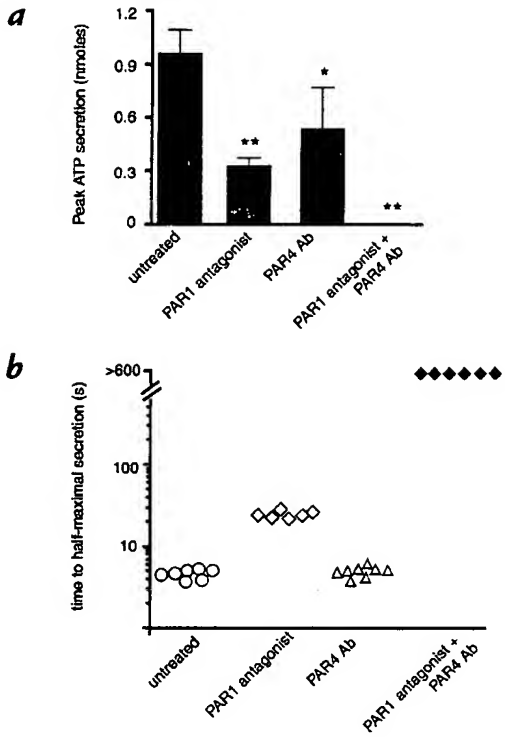


Figure 7

The effects of inhibition of PAR1 and/or PAR4 on platelet ATP secretion in response to thrombin. (*a*) Peak ATP secretion. Platelets were pretreated with buffer alone, PAR4 IgG (1 mg/ml), PAR1 antagonist BMS200261 (100 μM), or PAR1 antagonist plus PAR4 IgG as indicated, and then stimulated with 30 nM thrombin. Peak ATP concentration in the 10 min after addition of thrombin was measured by lumiaggregometry. Preimmune IgG had no effect (not shown). Data are mean ± SD (n = 5–7) of peak secretion measured; similar results were obtained with platelets from two individuals. Data were analyzed by two-way ANOVA and t test with a Bonferroni correction for multiple comparisons. *$P \cong 0.06$, **$P < 0.001$ compared with untreated group. Note that no secretion was detected during the 10 min after addition of 30 nM thrombin to platelets treated with PAR1 antagonist plus PAR4 IgG. (*b*) Time to half-maximal secretion. Time to reach 50% of the peak ATP secretion response elicited by 30 nM thrombin in each group (*a*) was measured. Platelets were pretreated with buffer alone (*open circles*), PAR1 antagonist (*open diamonds*), PAR4 IgG (*open triangles*), or PAR1 antagonist plus PAR4 Ab (*closed diamonds*) as in *a*, and then stimulated with 30 nM thrombin. Points displayed as >600 s indicate no measurable secretion within 10 min after addition of thrombin. PAR4 preimmune IgG had no effect inhibitory effect in such experiments, even in the presence of PAR1 antagonist (not shown).

gest that PAR3 has no important role in activation of human platelets by thrombin. Indeed, PAR3 mRNA and protein were not detected in human platelets but were readily detected in Dami cells. Such negative data regarding PAR3 expression in platelets must of course be interpreted with caution. Our failure to detect PAR3 mRNA and protein in human platelets is concordant with our functional data but at variance with a recent report of PAR3 expression in human platelets detected by RT-PCR and flow cytometry (26). In the latter study, the RT-PCR was not quantitative, and potential cross-reactivity of the PAR3 antiserum was not discussed, possibly accounting for our different results. Alternatively, platelets from only a handful of individuals have been studied, and it is possible that regulation or individual differences in PAR3 expression account for our differing results. Regardless, we know of no data that implicate PAR3 function in activation of human platelets by thrombin.

It is interesting to compare and contrast thrombin signaling in human and mouse platelets. This study shows that human platelets utilize both PAR1 and PAR4, with no apparent role for PAR3. By contrast, mouse platelets utilize PAR3 and PAR4 (17), with no apparent role for PAR1 (14). A definitive answer to whether additional receptors play a role in the mouse awaits generation of PAR3/PAR4 double knockout mice. In human platelets, PAR1 is necessary for responses to low concentrations of thrombin; in mouse platelets, PAR3 plays this role (17). Thus, despite the use of distinct receptors, platelets in both species use a dual thrombin receptor system in which a high-affinity receptor (PAR1 in human, PAR3 in mouse) mediates responses to low concentrations of thrombin and a low-affinity receptor (PAR4) mediates signaling at high concentrations. Interestingly, both PAR1 and PAR3 have obvious hirudin-like domains (3, 29). In PAR1, this domain binds thrombin's fibrinogen binding exosite and is critical for PAR1's efficient cleavage and activation by thrombin (3, 5, 29–32). PAR4 has no such domain, perhaps accounting for its slower cleavage by thrombin and right-shifted concentration response curve (17, 18).

The biological significance of having dual thrombin receptors in platelets remains uncertain. PAR3-deficient mice showed no spontaneous bleeding and had normal bleeding times; thus, in mice, the high-affinity receptor is not necessary for normal hemostasis when the low-affinity receptor is present. Whether different challenges will unmask a requirement for PAR3 in hemostasis or thrombosis is unknown, and whether combined deficiency in PAR3 and PAR4 will provoke a bleeding diathesis remains to be determined. It is possible that two receptors in platelets simply provide redundancy in an important system, but a variety of more interesting possibilities are apparent. It is possible that a capacity to respond to thrombin over a greater concentration range is important for reasons not yet understood. More broadly, PAR1, PAR3, and PAR4 might mediate responses to proteases or ligands other than thrombin or allow thrombin itself to activate distinct signaling pathways or to trigger signaling with varied tempos of activation or shutoff. The existence of multiple receptors also allows for distinct temporal and spatial expression. The finding of PAR3 expression in Dami cells (Figs. 1 and 2), but not in human platelets, is provocative in this regard. Dami cells were derived from a patient with megakaryoblastic leukemia and express a number of megakaryocyte markers (19). PAR3 is also expressed by HEL cells and K562 cells (data not shown, and ref. 26). These results raise the possibility that PAR3 might be expressed by hematopoietic cells in the erythroid/megakaryocyte lineage but extinguished in mature megakaryocytes and platelets. The role of PAR expression in hematopoiesis, if any, and whether PARs might serve as useful markers of differentiation, remains to be explored.

Because of the role of thrombin and platelet activation in myocardial infarction and other pathological processes, identifying and blocking the receptors by which thrombin

activates platelets has been an important goal. Iterations around PAR1's tethered ligand sequence SFLLRN have already led to the development of potent peptide-based antagonists (27). These antagonists blocked human platelet activation by SFLLRN itself and by low concentrations of thrombin but were ineffective at high concentrations of thrombin (27). This study strongly suggests that persistent platelet responses to high thrombin concentrations in the presence of a PAR1 antagonist were due to PAR4, which is not blocked by the antagonist. The PAR1 antagonist BMS200261 was in fact quite effective at blocking activation of PAR1 by high concentrations of thrombin (Fig. 5, and data not shown) but became effective at blocking platelet activation by high concentrations of thrombin only when PAR4 was blocked simultaneously.

The finding that PAR1 and PAR4 account for all, or virtually all, of the ability of human platelets to respond to thrombin should excite interest in the development of thrombin receptor antagonists as possible antithrombotic agents. Agents that inhibit signaling via the thromboxane and ADP receptors are effective antithrombotic drugs (33, 34). Given thrombin's remarkable potency as a platelet activator and its ability to activate even aspirin-treated platelets (35, 36), blockade of thrombin signaling in platelets might also prove to be a useful strategy for preventing thrombosis. Because inhibition of PAR1 alone markedly attenuated platelet responses at low concentrations of thrombin, PAR1 antagonism might be sufficient for an antithrombotic effect. In this scenario, PAR4 might ensure a minimal level of thrombin signaling and act as a safety buffer. Alternatively, it may be necessary to block both PAR1 and PAR4 to prevent thrombosis. Using genetically modified mice and inhibitor studies in other species, it will now be possible to determine if these strategies should be pursued.

Acknowledgments

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Species Variability in Platelet and other Cellular Responsiveness to Thrombin Receptor-derived Peptides

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Summary

The aggregation of platelets from a variety of animal species in response to thrombin receptor-derived activating peptides was evaluated. A series of 14-(SFLLRNPNDKYEPF), 7-(SFLLRNP-NH_2), 6-(SFLLRN-NH_2) or 5-(SFLLR-NH_2) residue peptides, the structures of which were based on the deduced amino acid sequence of the human thrombin receptor, promoted full aggregation of platelets in plasma from humans, African Green and Rhesus monkeys, baboons and guinea pigs at 4–50 μM depending on the peptide used. Platelets in plasma from rabbit, dog, pig, and hamster underwent a shape change but failed to aggregate in response to these peptides over 3 log units of peptide up to 800 μM, despite being fully responsive to human thrombin. However, because the receptor peptides induced shape change in the platelets from these non-aggregating species, they apparently can activate some of the intracellular signaling system(s) usually initiated by thrombin in these platelets. In contrast, platelets from rats did not undergo shape change or aggregate in response to the peptides. A 7-residue receptor-derived peptide based on the deduced amino acid sequence of the clone of the hamster thrombin receptor (SFFLRNP-N_2) was nearly as efficacious as the corresponding human receptor-derived 7-residue peptide to promote aggregation of human platelets. However, the hamster peptide could not promote aggregation of hamster platelets in plasma at up to 800 μM peptide, while a shape change response was elicited. Platelets from rats, rabbits and pigs also did not aggregate in response to this peptide derived from the hamster thrombin receptor, but all species except the rat underwent a shape change. Longer 17-residue peptides derived from the sequences of the hamster or mouse thrombin receptors elicited aggregation of human platelets but no aggregation of the hamster platelets. In contrast, the human 14- and 5-residue and the hamster 7-residue thrombin receptor-derived peptides promoted mitogenesis of CCL39 cells, a hamster fibroblast cell line. Finally, the human 6-residue thrombin receptor-derived peptide promoted contraction of normal and de-endothelialized canine coronary artery rings, despite having no pro-aggregatory effect on canine platelets. Taken together, these results demonstrate that the thrombin receptor-derived peptides are able to mimic many, but not all, of the activating effect of thrombin in different tissues from multiple species. The heterogeneity of responsiveness to these peptides should be taken into account in future experiments designed to elucidate the mechanism of thrombin stimulation of platelets and other cells.

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Introduction

The serine protease thrombin is the most potent physiological activator of platelets known. It directly activates platelets and other cells via one or more cell surface receptor(s) (1). The cloning (2, 3) of a thrombin receptor was recently described. Based on its primary sequence and on its characteristics of activation this receptor belongs to the family of 7 transmembrane G-protein linked receptors. According to the novel mechanism of activation of the receptor proposed by Coughlin et al. (2, 4) thrombin cleaves the extracellular domain of the receptor to create a new amino terminus, which can then act as a tethered ligand to activate the receptor. Peptides as short as the first 5 amino acids derived from the sequence of the proposed new amino terminus are able to fully activate human platelets and naive cells transfected with the clone for the thrombin receptor (5–8). Receptor activation on platelets results in the functional responses of platelet aggregation, secretion, Ca^{++} mobilization, protein phosphorylation, phosphatidyl inositol metabolism and inhibition of adenylate cyclase activity (9, 10). Thus, the receptor peptides can mimic many of the actions of thrombin itself in platelets.

Evidence for the existence of the cloned thrombin receptor has been presented for a wide variety of cells including endothelial cells, smooth muscle cells, mesenchymal-appearing cells and macrophages (reviewed in 4). In order to further explore the universality of this receptor and its mode of activation and role in normal hemostasis and thrombosis, we examined the effect of human thrombin receptor-derived peptides on the activation of platelets and other cells from a variety of animal species. These studies revealed that platelets from many species are not fully responsive to these receptor-derived peptides, despite being responsive to thrombin. However, other cells from these same species do give a full physiological response to the peptides.

Methods

Materials

Equine tendon type I collagen and ADP were from Chrono-Log Corp., Havertown, PA.; thrombin for the vascular reactivity studies was from Sigma, for the tissue culture studies it was from American Diagnostica, and for all other studies it was kindly provided by Dr. John W. Fenton II, Albany, N. Y.; human fibrinogen was from Calbiochem; CCL39 cells were obtained from the ATCC (Rockville, MD); peptides were from ABI; all other reagents were from Fisher and Sigma.

Platelet Preparation

Blood was collected from non-human species, or from healthy human volunteers free of aspirin and other drugs for at least 8 days, into 3.8% trisodium

citrate anticoagulant (1:10). The platelet rich plasma (PRP) was prepared by centrifugation and the platelet count was adjusted to 3×10^8 per ml with autologous platelet poor plasma. This PRP was used for all aggregations of platelets from the various animal species. For some studies human platelets were isolated by differential centrifugation and then washed in a modified Tyrode's buffer (5 mM HEPES, 0.3 MM NaH_2PO_4, 3 mM KCl, 134 mM NaCl, 5 mM glucose, 2 mM $MgCl_2$, 12 mM $NaHCO_3$, pH 6.5), containing 1 mM EGTA, 20 μg/ml apyrase and 3.5 mg/ml BSA as previously described (11). The final platelet suspension was at 2×10^8 platelets per ml in the same buffer at pH 7.4, without EGTA and apyrase.

Platelet Shape Change and Aggregation Assay

PRP or the washed platelets with 0.2 mg/ml human fibrinogen were incubated for 2 min at 37° C. Thrombin or a thrombin receptor-derived peptide was added to the sample in a Chronolog aggregometer and shape change and aggregation was monitored as a decrease in light transmittance, respectively. When thrombin was used as an agonist the PRP was pre-incubated for 2 min with 2 mM Gly-Pro-Arg-Pro peptide in order to prevent thrombin-mediated fibrin polymerization (12). The final amount of light transmittance (extent of aggregation) or the rate of change of light transmittance (rate of aggregation) was calculated by the aggrolink software provided by Chronolog Corp. with the aggregometer. In all cases qualitatively comparable results were obtained when either the rate or extent of aggregation was monitored.

Peptide Synthesis

Thrombin receptor peptides were synthesized as previously described (13). Briefly, peptide resins were assembled on solid support using an Applied Biosystems model 430A automated peptide synthesizer (t-BOC based chemistry). Peptides as carboxyl terminal amides (indicated by the symbol-NH_2) were synthesized on the benzylhydrylamine resin hydrochloride. The protected peptide-resins were treated with anhydrous liquid HF containing 10% anisole for 60 min at 0° C. The crude peptide products were purified by preparatory HPLC on a DELTA-PAK C_{18} column. Fractions containing product of at least 99% purity were combined and characterized by NMR and for amino acid composition after 6N HCl acid hydrolysis.

Cell Proliferation Assays

Cell culture of CCL39 cells and thymidine incorporation were carried out as previously described (13). Briefly, the cells were seeded in DME-10% FCS at 50,000 cells/well in cluster 24 plates (1 ml/well), grown to confluence, washed with phosphate buffered saline and exchanged into DME : Ham's F-12 (1:1) for growth arrest for 28 h. At this time the cells were exchanged into the same medium containing 1μCi/ml [3H]-methyl thymidine, 1 μg/ml cold thymidine and either 10% FCS, thrombin or thrombin receptor peptide. After 22 h the cells were washed with ice cold phosphate buffered saline, and treated with 1 ml 10% TCA for 10 min followed by rinsing with ethanol : ether (2 : 1), drying, solubilization and monitoring for radioactivity. Background, defined as the counts measured with exposure to medium only, was typically <8% that observed with FCS and was subtracted from all samples. Results are expressed as % thymidine incorporation relative to the FCS control in each experiment. All samples were tested in duplicate.

Vascular Smooth Muscle Tension Generation

Male purpose-bred mongrel dogs (9.2–10.3 kg) were anesthetized with sodium pentobarbital (35 mg/kg, i.v.) and mechanically ventilated with room air (Harvard Apparatus). A left thoracotomy was performed and the heart was excised and immersed in oxygenated, warmed (37° C) physiological salt solution (PSS: 112 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.0 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.6 mM dextrose and 1.1 μM ascorbic acid). The buffer was equilibrated at 37° C with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. The left circumflex artery was dissected free from the heart, blood was rinsed from the lumen, connective tissue and fat were carefully removed and the vessel was sectioned into rings of 2 mm in length. For some of the experiments, the endothelium was removed by inserting a 23 gauge needle adapter into the ring and rolling it gently on a moistened paper towel. To record force generation, each ring was placed between two stainless-steel wires within individual tissue bath chambers containing 15 ml of 37° C oxygenated PSS. The lower wire was immobilized while the upper wire was connected to a Statham UC2 transducer. Each ring, with or without endothelium, was maintained at a baseline force of 1 gram during an equilibration period of 60 min. Tissues were primed with 40 mM KCl (2 times), following the wash-out after each KCl stimulation, baseline force was re-established at 1 gram. Arterial rings were then evaluated for their response from baseline to 1.0 μM of the thrombin receptor-derived activating peptide, SFLLRN-NH_2, or for the effect of 100 nM thrombin on vessels precontracted with 3 nM endothelin. Adequate de-endothelialization was confirmed by the addition of acetylcholine (0.1 μM) to the contracted preparations. In separate experiments, the response to cumulative doses of the peptide was demonstrated. Isometric tension data were collected on line at 2 s intervals and stored on a Compaq (Proline 4/33) computer system with software provided by Branch Technology. Digital data and a real time tracing were continuously displayed on a Flex Vision color monitor (model PMV/1448) with a hard copy provided by a Hewlett Packard laser jet PCL5. Hard copy analog data were continously recorded on a 8-channel Hewlet Packard 7758A.

Results

Aggregation of Platelets from Various Species in Response to Thrombin Receptor-derived Peptides

A systematic evaluation of platelet aggregation provoked by 5-residue (SFLLR-NH_2) or 14-residue (SFLLRNPNDKYEPF) peptides derived from the sequence of the human thrombin receptor was undertaken in platelet rich plasma from a variety of different animal species. As shown in Table 1, of the 9 different species evaluated only platelets from primates and those from the guinea pig demonstrated a full aggregation response. Platelets from a variety of species commonly used in thrombosis studies, including dog, hamster, rabbit, and pig showed a shape change but failed to aggregate in response to up to 800 μM peptide. Representative responses are shown in Figs. 5 and 7 as discussed below. Rat platelets did not aggregate and also did not change shape in response to the peptides. In contrast, only 1–10 μM of these peptides were required to promote full aggregation of human platelets. Receptor-derived peptides of intermediate length also failed to induce

Table 1 Effect of peptides derived from the human thrombin receptor on the aggregation of platelets from various animal species

Species	Aggregation[a]	Shape change
Human	87 ± 5	YES
Baboon	70 ± 4	YES
African green monkey	57 ± 9	YES
Rhesus monkey	68 ± 12	YES
Rabbit	3 ± 1	YES
Dog	0	YES
Pig	14 ± 2	YES
Rat	0	NO
Hamster	0	YES
Guinea pig	65 ± 13	YES

[a] Platelet rich plasma was challenged by up to 800 μM SFLLRN-NH_2, SFLLRN-NH_2 or SFLLRNPNDKYEPF peptides and aggregation and shape change monitored as described in Methods. Aggregation values are expressed as the maximal extent of aggregation observed ± the standard deviation. Results are from 3–6 separate experiments for each species.

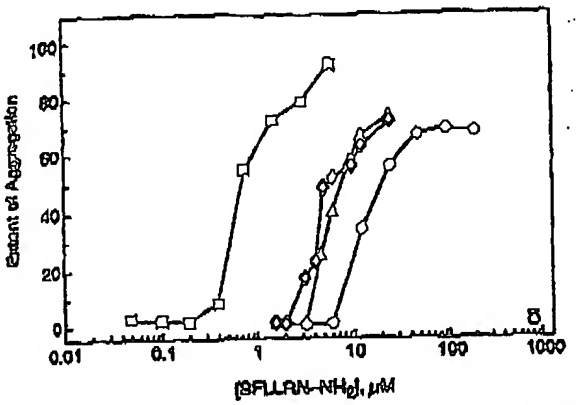

Fig. 1 Human thrombin receptor peptide stimulated platelet aggregation. PRP from the indicated species were challenged with the human thrombin receptor-derived peptide, SFLLRN-NH_2, and aggregation monitored as described in Methods. These results are the average of 3 separate experiments for each species, human (□), guinea pig (△), Rhesus monkey (◇), baboon (○), dog (▽) and hamster (○)

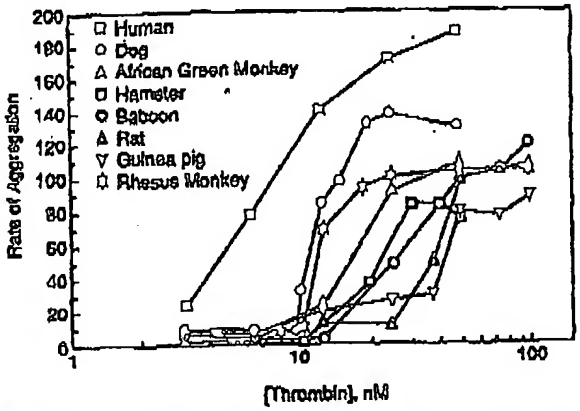

Fig. 2 Thrombin-stimulated platelet aggregation. PRP (0.25 ml) from the indicated species was prepared and stimulated by the indicated concentrations of human thrombin and aggregation monitored as described in Methods. These results are the mean rate of aggregation for human (□), n = 4; dog (○), n = 2; African green monkey (△), n = 2; hamster (□), n = 1; baboon (○), n = 2; rat (△), n = 3; guinea pig (▽), n = 2; and Rhesus monkey (▷), n = 2

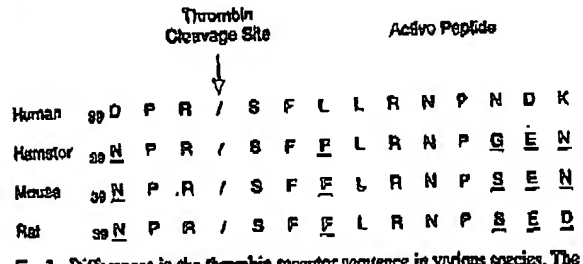

Fig. 3 Differences in the thrombin receptor sequence in various species. The third residue of the activating peptide from the hamster (3), mouse (14), and rat (15) has an F (Phe) substitution for L (Leu). Underlined residues indicate differences from the human sequence (2)

Table 2 Platelet aggregation of non-human, hamster, pig and rat, platelets in plasma or human washed platelets was carried out as described in Methods. The results are from at least 3 separate experiments for each peptide in each species

Peptide	Aggregation EC_{50}, μM	
	Non-human platelets	Human platelets
SFLLR-NH_2	>800	1
SFLAR-NH_2	>800	15
SFALR-NH_2	>800	1.3
A(3Qual)LLR-NH_2°	>800	>800
A(hF)LLR-NH_2°	>800	>800

° 3Qual = 3-(3quinolyl)alanine, hF = homo phenylalanine.

aggregation (data not shown). Platelets from the responding species of guinea pigs, Rhesus monkeys and baboons, however, fully aggregated in response to the human receptor peptides, although 10 to 40-fold more peptide than that needed to promote aggregation of human platelets was required for an equivalent response as shown in Fig. 1.

The platelets from the species which did not aggregate in response to human thrombin receptor-derived activating peptides, did aggregate after stimulation by human thrombin as shown in Fig. 2, and by ADP and collagen, two other platelet agonists which act via distinct receptors (not shown). This result demonstrates that the platelets from these species can aggregate and that they have thrombin receptors that are recognized and activated by human thrombin.

A comparison of the sequences of the thrombin receptors deduced from cDNA clones of the thrombin receptor from human (2), hamster (3), mouse (14) and rat (15) sources is shown in Fig. 3. In the proposed activating peptide region there is a substitution of a Phe for Leu at position 44 in the rodent species. We speculated that activating peptides with this substitution might activate platelets from homologous species, that had not responded to the human receptor-derived peptides. A 7-residue peptide incorporating this substitution was prepared and its effect on the aggregation of human platelets compared to that elicited by the

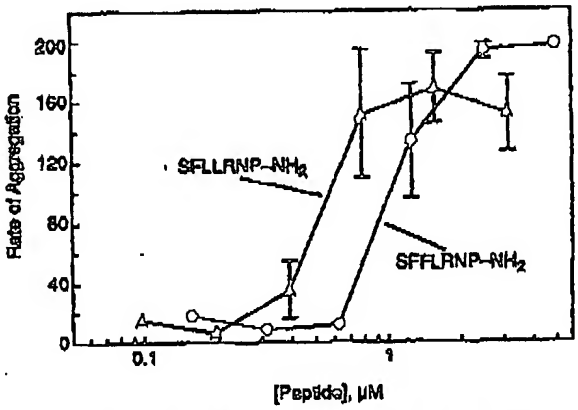

Fig. 4 Peptide-stimulated human platelet aggregation. Washed human platelets were stimulated by 7-residue peptides based on the sequence of the human thrombin receptor, SFLLRNP-NH_2 (△), or the hamster thrombin receptor, SFFLRNP-NH_2 (○), and aggregation monitored as described in Methods. The results are expressed as mean ± S. E. for 3 separate experiments for each peptide

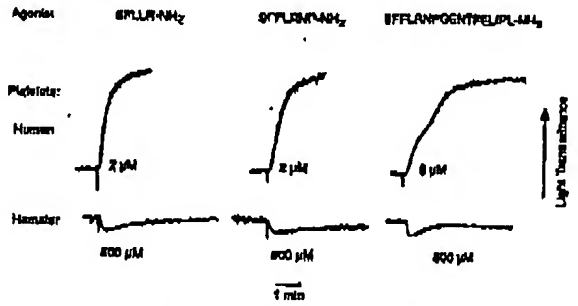

Fig. 5 Aggregation of human and hamster platelets. Human or hamster PRP was stimulated by the indicated concentration of human thrombin receptor-derived peptide, SFLLR-NH_2, or the hamster thrombin receptor-derived peptides, SFFLRNP-NH_2 and SFFLRNPGENTFELIPL-NH_2, and aggregation monitored as described in Methods and as shown by the tracings. These results are representative of 3–5 separate experiments

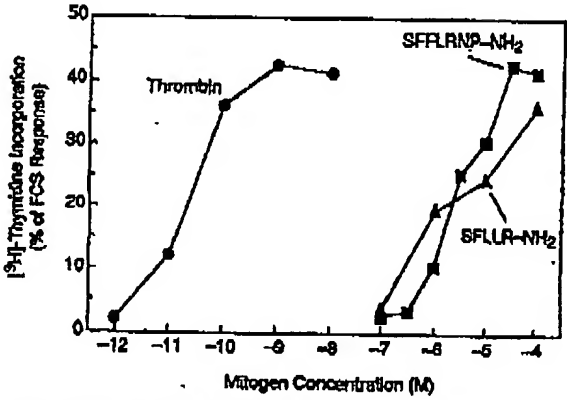

Fig. 6 Mitogenic activity of thrombin receptor agonist peptides towards CCL39 cells. (●) human thrombin, (▲) a human thrombin receptor-derived peptide, SFLLR-NH_2, or (■) the hamster thrombin receptor-derived peptide, SFFLRNP-NH_2, were added to confluent cultures of growth arrested CCL39 cells and after 22 H the extent of DNA synthesis was determined by measuring [³H]-Thymidine incorporation into newly synthesized DNA as described in Methods and (13). The data are expressed as [³H]-Thymidine incorporation as a percentage of the response to 10% fetal calf serum (FCS) in the same experiment

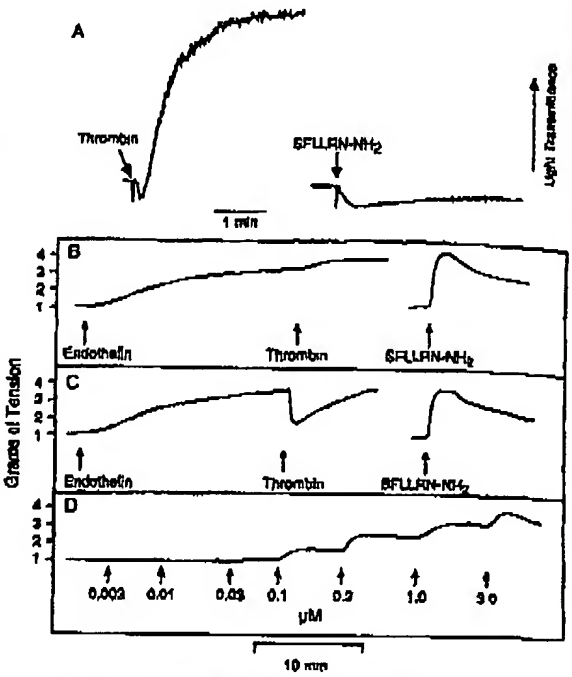

Fig. 7 Effect of thrombin and a thrombin receptor peptide on canine tissues. (A) Aggregation of dog PRP to 25 nM thrombin or 800 μM SFLLRN-NH_2 peptide was carried out as described in Methods. Vascular response of canine coronary artery rings without (B) and with endothelium (C, D) to 100 nM thrombin or 1 μM SFLLRN-NH_2 peptide carried out as described in Methods. (D) Dose-related response of canine coronary vessel rings to SFLLRN-NH_2 peptide added cumulatively. Tracings of B-D are representative of 4–5 separate experiments

corresponding 7-residue human receptor peptide. As shown in Fig. 4 the rodent-derived receptor peptide also promoted full aggregation of human platelets similarly to that induced by the human receptor-derived peptide.

The effect of the hamster receptor-derived peptide together with that of the human receptor-derived peptide of the same length on hamster platelet aggregation is illustrated in Fig. 5. The human platelets in plasma showed comparable aggregation in response to 2 μM of each peptide, however, both the human- and rodent-derived peptides at up to 800 μM failed to promote aggregation of hamster platelets despite the latter being from the same species. In order to rule out the possibility that a 7 residue hamster receptor peptide is of insufficient length to activate the hamster platelets, a longer peptide of 17 amino acids derived from the cleavage site of the hamster receptor clone was prepared. This peptide at up to 800 μM also did not induce aggregation of the hamster platelets despite being a potent activator of human platelet aggregation as shown in Fig. 5. Stimulation of rodent and pig platelets by 5-residue peptides with an alanine substitution in position 3 or substitutions in the 2 and 4 positions with other residues in an attempt to mimic their endogenous receptor ligands also did not result in peptides which could initiate aggregation of these platelets as shown in Table 2.

In order to determine if peptides were being inactivated by some plasma component both human receptor-derived activating peptides of 5 and 14 amino acids and the 7 and 17 amino acid rodent-derived peptides were incubated with plasma from nonaggregating species for 5 min. at 37° C and then added to human platelets at 2 times their EC_{50} concentration to determine if the plasma from these species was inactivating the peptides. These platelets aggregated with a response comparable to that observed in response to the same concentration of peptide not pre-incubated with the plasma. Thus, there was no peptide inactivation in the plasma from these non-responding species under these conditions.

Mitogenic Response of CCL39 Cells

We had previously observed that thrombin and human thrombin receptor peptides could promote increased incorporation of [³H]-thymidine in CCL39 cells, a hamster-derived fibroblast cell line (13). Since hamster platelets did not aggregate in response to either human or hamster thrombin receptor-derived peptides it was of interest whether

or not hamster fibroblasts fully respond to the hamster-derived peptides. As shown in Fig. 6 and as previously reported (13), thrombin and both human and hamster-derived receptor peptides induced a strong mitogenic response in CCL39 cells. Although the potencies of each to elicit these responses was different, the same final extent of [^{3}H]-thymidine was incorporated in response to each stimulus.

Effect of Thrombin and a Thrombin Receptor Peptide on Canine Vascular Response

We next explored whether the apparent tissue selective responsiveness to thrombin receptor-derived peptides was unique to the hamster or if thrombin responsive tissues in other species could respond to these peptides. Dog platelets did not aggregate in response to human thrombin receptor-derived peptides (Fig. 1 & 7A) but they did aggregate to thrombin stimulation (Fig. 2). The effect of thrombin and the human thrombin receptor-derived peptide, SFLLRN-NH_2, on isolated canine coronary artery rings was evaluated. Thrombin induced a moderate contraction (22.4% or 0.48 g increase) of 3 nM endothelin precontracted (2.14 g) de-endothelialized rings (Fig. 7B), and elicited a marked relaxation (72.1% or 1.78 g decrease) of endothelin precontracted (2.47 g) coronary artery rings with endothelium from the same vessel (Fig. 7 C). The endothelin-stimulated contraction (3 g active tension) was similar to that typically elicited by 40 mM KCl (85% of the KCl response in the denuded rings and 65% of the KCl response in the intact vessel segments). In contrast to its effect on canine platelets, the 6-residue thrombin receptor peptide elicited a dose-related contraction above baseline tension levels in the canine coronary artery rings both without (Fig. 7 B) and with endothelium (Fig. 7 C & D). The magnitude of these contractions were 2.95 g or 95% of the response to 40 mM KCl without endothelium and 2.76 g or 75% of the response to KCl in the same vessel with intact endothelium.

Discussion

Thrombin plays a vital role in many aspects of normal hemostasis (reviewed in 16). It is a procoagulant enzyme, mediating the conversion of fibrinogen to fibrin and the activation of Factors V and VIII, thereby promoting its own production. Thrombin can also act as a feedback inhibitor of its own production by activating the natural anticoagulant, protein C (17). It also directly activates platelets and many other cell types via a cell surface receptor(s) (1). With the cloning, expression and elucidation of the mechanism of activation of a human thrombin receptor on megakaryocytic-like cells (4) it became possible to directly examine activation of this thrombin receptor on platelets and other cells without interference from the other responses to thrombin.

According to the proposed mechanism of activation of this receptor (4) peptides derived from the newly created amino terminus should activate platelets directly via this receptor. In the current studies we examined the ability of peptides derived from the amino acid sequence deduced from the clone of the human or rodent thrombin receptor to promote aggregation of platelets from a variety of different species. We found that in addition to human platelets only those from primates and the guinea pig (Table 1) fully aggregated in response to these peptides. This result is in agreement with the findings of Kinlough-Rathbone et al. (18) and Catalfamo et al. (19). The non-aggregating platelets from the dog, rat, rabbit and hamster, however, were physiologically competent as they did aggregate in response to other agonists such as ADP or collagen. They also have a thrombin receptor, as they aggregated in response to human thrombin (Fig. 2). The platelets are partially activated by the peptides derived from the thrombin receptor, as the platelets from all species tested (except the rat) underwent a shape change in response to these peptides.

The precise mechanistic basis for the inability of the peptides with sequences derived from the human thrombin receptor to promote full aggregation of platelets from the non-primate species is unknown. It is unlikely that the inability of the human receptor peptides to promote full aggregation of platelets from many species is due simply to a lower affinity of the same thrombin receptor for these peptides (all of which fully respond to thrombin). Kinlough-Rathbone et al. (18) tested the effect of 100 μM of the thrombin receptor peptide with the animal species, a 20-fold increase over the amount required to promote full aggregation of human platelets. In the current studies an 800-fold excess of peptide over that which induced full aggregation of human platelets in plasma was unable to elicit aggregation of platelets from non-responding species. The lack of aggregation in response to a concentration of peptide almost three orders of magnitude greater than that required to promote full aggregation of human platelets suggests that the receptors are pharmacologically distinct between species. Likewise, because shape change and not aggregation was provoked by the receptor peptides in several non-primate species, pharmacologically distinct thrombin receptors within these species receptors may mediate these responses.

An alternative explanation for the lack of aggregation of platelets from many species could be explained by a high amount of aminopeptidase M activity in plasma from the non-responding species which would cleave the amino terminus serine from the activating peptides, thereby rendering them inactive as described by Coller et al. (20). This is however, unlikely, because incubation of the peptides with platelet rich plasma from the various non-responding species did not render them incapable of promoting aggregation of human platelets (not shown). In addition, as the peptide concentration examined (800 μM) the peptidase activity would have to hydrolyze more than 99.9% of the peptide within a few seconds.

The lack of aggregation response of platelets from some species to the receptor peptides is unlikely due to a difference in thrombin receptors that is unique to the non-responding species or to platelets within a given species. It could be argued that the platelets from the non-responding species do not aggregate because the sequence of their thrombin receptor in the region of the tethered ligand is different at some critical residue, and therefore, their activating peptide would be different than that of the human-derived peptide. Structure activity studies (5-8, 21) indicate that the second residue of the activating against peptides is the most critical for agonist activity. Several peptides at up to 800 μM with substitutions in this residue, and in residues 3 and 4 did not promote platelet aggregation of the non-responding species, while being effective agonists for human platelet aggregation (Table 2). Cloning of the thrombin receptor from rodents, including hamster (3), mouse (14), and rat (15), revealed a substitution of a Phe for Leu at the third residue. Peptides of 5 to 14 residues in length derived from the hamster receptor sequence stimulated the aggregation of human platelets, but the effect of this peptide on platelets from other species, including hamster, was not previously reported (22). We found that a 7 or 17 residue hamster receptor peptide was nearly as effective as the human sequence to promote aggregation of human platelets, but at up to 800 μM did not provoke aggregation of platelets from hamster the species from which these peptides were derived (see Fig. 3). The lack of a response of the hamster platelets to the hamster-derived sequence is inconsistent with this simple explanation and suggests a more complex mechanism of receptor activation.

In the current studies, peptides derived from the human thrombin receptor could not promote aggregation of hamster platelets, yet previous studies had demonstrated that these peptides could either promote phosphatidyl inositol turnover and inhibition of adenylate cyclase (22) or mitogenesis (13, 23) in CCL 39 cells, a hamster fibroblast cell line. The current studies confirm the ability of human peptides to promote mitogenesis in these cells and demonstrate that a hamster-derived peptide also promotes mitogenesis of these hamster fibroblast cells. Also, we found that human receptor peptides stimulated smooth muscle contraction in coronary artery rings from dogs, despite not being able to induce aggregation of their platelets. Previous work had demonstrated endothelium-dependent relaxation of canine saphenous veins and contraction of dog coronary artery strips by human thrombin receptor peptides (24). Rat platelets also did not aggregate in response to the human receptor peptides. Kinlough-Rathbone et al. (18) and Catalfamo et al. (19) also reported a similar finding. Yet, despite the lack of any rat platelet functional response to 800 μM human or rodent receptor peptide, human peptides have been shown to have an effect on vascular reactivity of rat tissues (25–27) and to be mitogenic for rat vascular smooth muscle cells in culture (28). Therefore, in at least these three species the thrombin receptors on non-platelet cells have different pharmacological properties than those on their platelets.

Although the molecular basis of this pharmacological difference in receptors is not yet known, these results suggest two possibilities for further exploration. First, there may be more than one thrombin receptor as has been previously suggested (reviewed in 29); the cloned receptor on primate and hamster platelets, which responds to both thrombin and receptor-derived peptides, and another receptor(s) on platelets from non-primate species that is molecularly unrelated and only fully activated by thrombin. The currently available molecular biological approaches would identify only receptors structurally related to the cloned receptor. Data from several other types of approaches do support the existence of more than one thrombin receptor. First, platelet activation induced only by lower concentrations of thrombin is antagonized by antibodies against the exosite region of the cloned thrombin receptor (30-32) and by PPACK-thrombin (33). Higher concentrations of thrombin can overcome this inhibition and thus may interact with a distinct receptor. Secondly, Seiler et al. (34) reported on two types of responses to thrombin in human platelets and suggested the existence of more than one receptor. One response was to low concentrations of alpha thrombin but not gamma thrombin and it was desensitized by receptor-derived peptides, while the other was to higher concentrations of alpha thrombin and to gamma thrombin and was not desensitized by the receptor peptides. A variety of thrombin binding proteins have been identified on platelet surfaces and have been implicated as being important for thrombin to promote activation in platelets and other cell types (35). Indeed, thrombin has been shown to bind to human platelets with at least three different affinities (36). The second possibility is that the receptor peptides may be partial agonists of the platelets from the non-aggregating species. In most cases the human and hamster-derived peptides could evoke a shape change response of the platelets. This suggests that a receptor interaction that initiates intracellular signaling sufficient to promote shape change but not aggregation in these species is occurring. Indeed, only the platelets from the rat showed no response to the peptides and pig platelets actually aggregated ~13% of full aggregation. Kinlough-Rathbone et al. reported that pig platelets fully aggregated in response to human peptides but those from rabbit and rat did not (18). The receptor which responds to the peptides may couple to different effector systems in the non-responding cells that do not allow, for example, the expression of competent fibrinogen receptors or some other requirement for aggregation. The further comparison of the effects of these peptides on platelets from primate and non-primates may allow for the distinction between intracellular events that elicit only shape change and those that elicit shape change and aggregation as induced by thrombin.

Acknowledgements

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were moved close to a perfusion pipette containing standard extracellular solution supplemented with Cu(II)(1,10-phenanthroline)$_3$ at different concentrations. A Cu(II)(1,10-phenanthroline)$_3$ stock solution was made by dissolving 150 mM $CuSO_4$ and 500 mM 1,10 phenanthroline in 4:1 water/ethanol. After reaching steady-state conditions, cells were moved to another perfusion pipette with standard extracellular solution containing 1 mM DTT. Current modifications under oxidizing and reducing conditions were observed as described for the MTS experiments. The time course was fit with a single exponential giving the time constant of modification.

To determine block by intracellular Cd^{2+}, Cd^{2+} was added in variable concentration to a modified intracellular solution devoid of EGTA containing (in mM): NaCl 140, $MgCl_2$ 2, HEPES 10, pH 7.4. Initially, the voltage dependence of the instantaneous current amplitude after a 300-ms prepulse to +75 mV was obtained on inside-out patches in standard intracellular solution. Then the Cd^{2+}-containing solution was applied by moving the patch into the stream of a perfusion pipette. The time course of the block was determined by repetitive pulsing. After reaching a steady-state value, the voltage dependence of the instantaneous current amplitudes was measured with the patch pipette in the solution stream and shortly after switching to standard intracellular solution. Relative block was obtained by dividing the current amplitude at the end of the prepulse before and after Cd^{2+} application.

Data were analysed with a combination of PClamp (Axon Instruments) and SigmaPlot (Jandel Scientific) programs. All data are shown as means ± s.e.m.

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A dual thrombin receptor system for platelet activation

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Platelet-dependent arterial thrombosis triggers most heart attacks and strokes. Because the coagulation protease thrombin is the most potent activator of platelets[1], identification of the platelet receptors for thrombin is critical for understanding thrombosis and haemostasis. Protease-activated receptor-1 (PAR1) is important for activation of human platelets by thrombin[2–6], but plays no apparent role in mouse platelet activation[7–9]. PAR3 is a thrombin receptor that is expressed in mouse megakaryocytes[10]. Here we report that thrombin responses in platelets from PAR3-deficient mice were markedly delayed and diminished but not absent. We have also identified PAR4, a new thrombin-activated receptor. PAR4 messenger RNA was detected in mouse megakaryocytes and a PAR4-activating peptide caused secretion and aggregation of PAR3-deficient mouse platelets. Thus PAR3 is necessary for normal thrombin responses in mouse platelets, but a second PAR4-mediated mechanism for thrombin signalling exists. Studies with PAR-activating peptides suggest that PAR4 also functions in human platelets, which implies that an analogous dual-receptor system also operates in humans. The identification of a two-receptor system for platelet activation by thrombin has important implications for the development of antithrombotic therapies.

PAR3-deficient mice (Fig. 1) developed normally and showed no spontaneous bleeding. Haematocrit, platelet counts and bleeding times[11] were indistinguishable from those of wild-type mice (data not shown). However, thrombin responses in PAR3-deficient platelets were markedly abnormal (Fig. 2). Wild-type platelets secreted their granule contents and aggregated reliably to 1 nM thrombin. In contrast, PAR3-deficient platelets were virtually unresponsive to 1 and 3 nM thrombin; 10 nM thrombin elicited delayed responses from PAR3-deficient platelets (Fig. 2) and the level of secretion eventually achieved by PAR3-deficient platelets was decreased compared with that in wild-type platelets. Even at 30 nM thrombin, the secretion response in PAR3-deficient platelets was delayed, but the level of secretion ultimately reached was comparable to that seen in wild-type platelets (Fig. 2). Secretion and aggregation in response to U46619, an agonist of the thromboxane receptor, were indistinguishable in wild-type and PAR3-deficient platelets even at submaximal agonist concentrations (data not shown). Thus PAR3 is necessary for normal responsiveness to thrombin in mouse platelets.

However diminished, thrombin responses did persist in PAR3-deficient platelets. What mediates these responses? The prototypical thrombin receptor PAR1 (ref. 2) plays no role in activation of mouse platelets by thrombin[9], and PAR3-deficient platelets, like wild-type platelets[7–9], did not respond to PAR1-activating peptide (data not shown). Persistent thrombin signalling in PAR3-deficient platelets therefore suggested the presence of an as yet uncharacterized thrombin receptor—perhaps another PAR family member. A GenBank BLAST search for PAR-related sequences revealed an EST (for expressed sequence tag) encoding an 11-amino-acid sequence that was 73% identical to the cognate sequence in PAR2. Because this region is conserved among PARs, a full-length complementary DNA was obtained. This cDNA (GenBank accession

number AF080215) encoded a 397-amino-acid protein, now designated PAR4, that was most closely related to human PAR3, with 30% amino-acid sequence identity. Proteases activate PARs by cleaving their amino-terminal exodomains to unmask a new amino terminus that then serves as a tethered ligand, binding intramolecularly to the body of the receptor to effect transmembrane signalling[2,12,13]. Examination of PAR4's amino-terminal exodomain revealed a putative thrombin cleavage site (Fig. 3) that was identical to the thrombin cleavage site in rat PAR1 (ref. 14). Expression of PAR4 in *Xenopus* oocytes did confer robust signalling to thrombin, but, at least in this system, PAR4 required higher thrombin concentrations than did the well-studied thrombin receptor PAR1 (Fig. 3). Thrombin cleaved a Flag epitope fused to PAR4's amino terminus[15] from the surface of PAR4-expressing oocytes, and active site-inhibited PPACK thrombin[16] did not cause cleavage or signalling (data not shown), which is consistent with a proteolytic activation mechanism. PAR4 signalling was relatively thrombin-specific; among the related arginine/lysine-specific proteases tested, only thrombin and trypsin elicited significant responses (Fig. 3).

Synthetic peptides that mimic the tethered ligands of PAR1 and PAR2 function as agonists for their respective receptors and have been used as pharmacological tools to probe the function of these receptors in various cell types[2,5,6]; the cognate peptide for PAR3 appears to be insufficiently active to function as a free ligand[10]. The location of the putative thrombin cleavage site in PAR4 predicted that the sequence GYPGKF would serve as PAR4's tethered ligand, implying that a synthetic peptide of the same sequence might function as a PAR4 agonist. As predicted, this peptide activated PAR4-expressing *Xenopus* oocytes but not uninjected or water-injected oocytes (Fig. 3 and data not shown). In the thrombin receptors PAR1 and PAR3, the phenylalanine in the second position of the tethered ligand (a tyrosine in the PAR4-activating peptide) is critical for the function of this domain[5,6,10]. Accordingly, the synthetic peptide GAPGKF had no activity at PAR4 (Fig. 3).

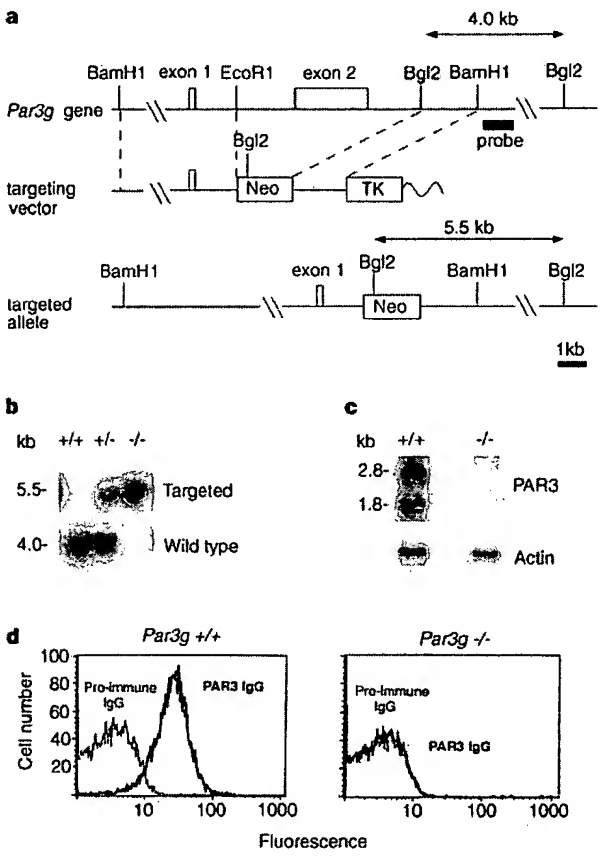

Figure 1 Generation of PAR3-deficient mice. **a**, Gene-targeting strategy. A replacement vector[26] was used to substitute a neomycin phosphotransferase expression cassette (Neo) for *Par3g* exon 2, which encodes the entire PAR3 protein except for its signal peptide. Wavy line represents plasmid backbone; TK, HSV thymidine kinase expression cassette. **b**, Southern-blot analysis of *Bgl*II-digested genomic DNA from the tails of pups derived from $Par3g^{+/-}$ matings using 3′ flanking probe (**a**). Targeting removed an endogenous *Bgl*II site and introduced a new *Bgl*II site. The 4.0-kb and 5.5-kb bands correspond to wild-type and targeted alleles, respectively. **c**, Northern-blot analysis of $Par3g^{+/+}$ and $Par3g^{-/-}$ mouse spleen mRNA using *Par3g* exon 2 probe and β-actin probe to control for lane loading. **d**, Flow cytometric analysis of wild-type and $Par3g^{-/-}$ platelets for PAR3 protein. Platelets were incubated with preimmune IgG (narrow line) or PAR3 IgG (wide line). Bound IgG was detected with FITC-labelled 2° antibody[23]. Each figure represents an analysis of 10,000 events. Note lack of surface PAR3 in $Par3g^{-/-}$ platelets.

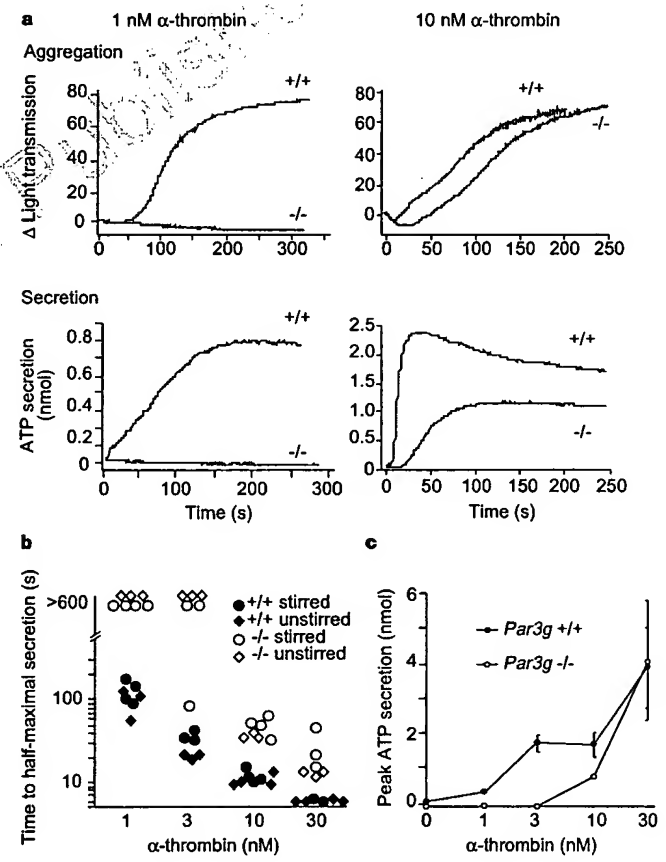

Figure 2 Thrombin responses in PAR3-deficient platelets. **a**, Aggregation and secretion of wild-type ($Par3g^{+/+}$) and PAR3-deficient ($Par3g^{-/-}$) platelets to 1 nM (left) and 10 nM (right) α-thrombin. Platelets were stirred and exposed to thrombin at 0 s. Similar results were obtained with five separate platelet preparations. **b**, Time to half-maximal secretion. $Par3g^{+/+}$ (filled symbols) and $Par3g^{-/-}$ (open symbols) platelets were stirred (circles) or left unstirred (diamonds) in a lumiaggregometer. Time to 50% of the peak response elicited by each of the indicated concentrations of thrombin was measured. Unstirred platelets did not aggregate. At each thrombin concentration, each point represents the response of a separate platelet preparation. Points at >600 s had no measurable secretion at 10 min. **c**, Dose response of $Par3g^{+/+}$ (filled circles) and $Par3g^{-/-}$ (open circles) platelets. Unstirred platelets were exposed to various concentrations of thrombin and peak ATP secretion was measured. Values are the mean responses (± s.e.m.) of three separate platelet preparations. Some error bars are obscured by the symbols.

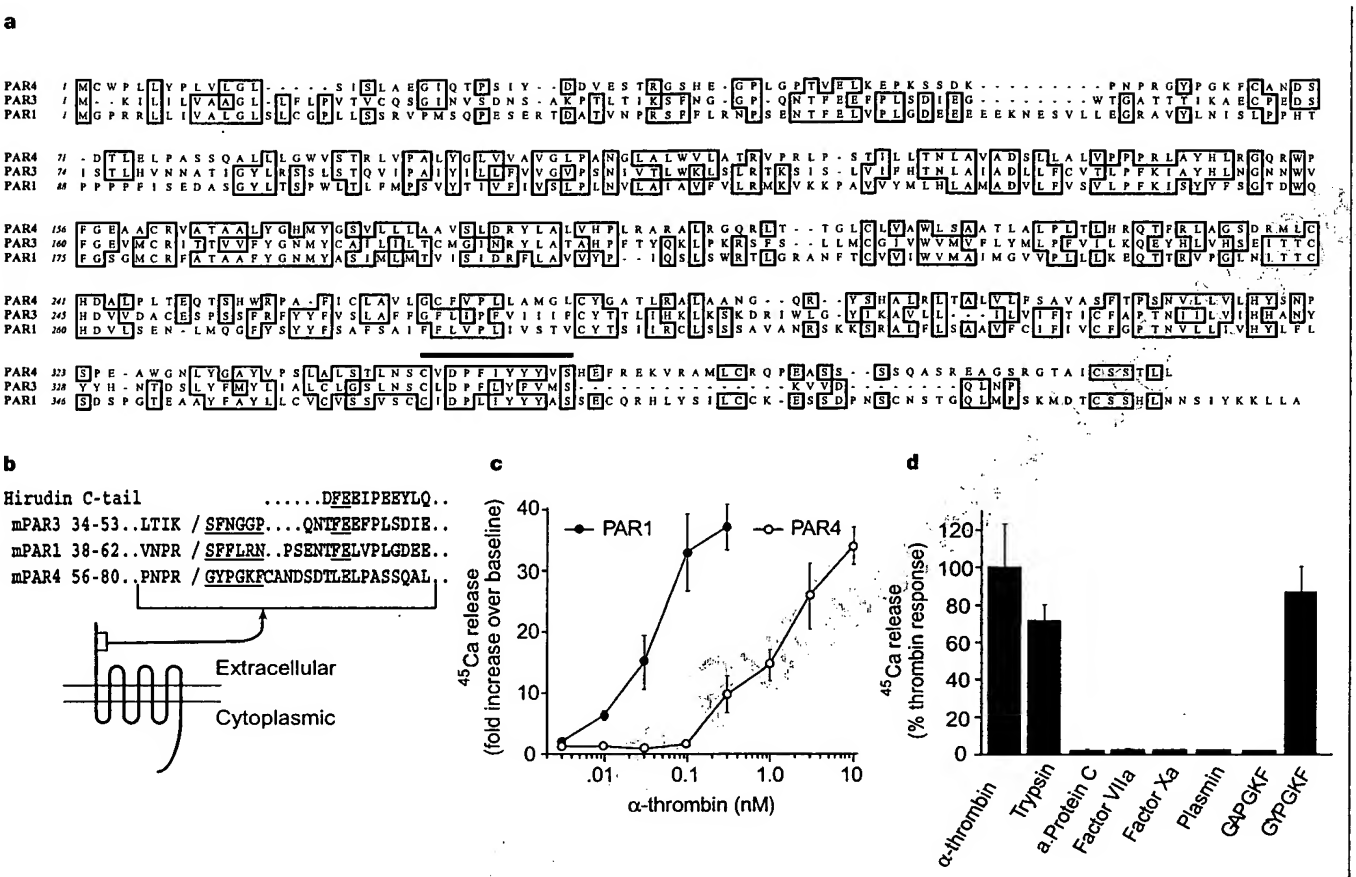

Figure 3 PAR4 amino-acid sequence and signalling properties. **a**, Sequence of PAR4 aligned with PAR3 and PAR1, the two known mouse thrombin receptors. Regions of amino-acid identity are boxed. The 11 amino acids of EST 400689 are overlined. **b**, Features of the amino-terminal exodomains of mouse thrombin receptors. The predicted thrombin cleavage site in PAR4 (/) is aligned with the known sites in PARs 1 and 3. The predicted or known tethered ligand sequences that are unmasked by receptor cleavage are underlined, as are amino-acid sequences resembling hirudin's carboxyl tail. The latter sequence in human PAR1 binds to thrombin's fibrinogen-binding exosite in a similar way to hirudin's C-tail[12,26,27]. **c**, Thrombin activation of PAR4 and PAR1. Agonist-triggered ^{45}Ca release was measured in *Xenopus* oocytes expressing mouse PAR4 and human PAR1 tagged at their amino termini with a FLAG epitope[15]. Data are mean ± s.e.m. ($n = 4$) and are expressed as the fold increase over baseline for each receptor. Surface expression of PAR4 measured with anti-FLAG monoclonal antibody was 1.8 times that of PAR1. Similar signalling data were obtained with untagged receptors. **d**, Specificity of PAR4 activation by proteases and peptides. ^{45}Ca release by oocytes expressing PAR4 was measured in response to the indicated active proteases (all at 5 nM except trypsin (0.5 nM)) or to the synthetic peptides GYPGKF and GAPGKF (each at 500 μM). Uninjected and buffer-injected oocytes did not respond to proteases or peptides at these concentrations. Data are mean ± s.e.m. ($n = 3$) and are expressed as a percentage of the maximum response to thrombin. In the experiment shown, thrombin caused a 33-fold increase in ^{45}Ca release. These experiments were replicated at least twice.

Taken together, these data show that PAR4 is a functional protease-activated receptor that is capable of mediating thrombin signalling.

Northern-blot analysis of mouse tissues revealed a relatively high level of expression of PAR4 in spleen, trace expression in heart, lung, skeletal muscle and kidney, and no detectable expression in brain, liver or testis (data not shown). *In situ* hybridization of spleen and bone marrow revealed that, like PAR3 (ref. 10), PAR4 mRNA is expressed in mouse megakaryocytes (Fig. 4). Finally, GYPGKF, a PAR4-activating peptide, activated both wild-type and PAR3-deficient mouse platelets, whereas the mutant GAPGKF peptide had no activity (Fig. 4 and data not shown). These data suggest that PAR4 functions in both wild-type and PAR3-deficient mouse platelets and is likely to be responsible for the residual thrombin signalling seen in the latter.

The characterization of a dual thrombin receptor system in mouse platelets leads to the question of whether a similar system operates in human platelets. Unlike mouse platelets, thrombin signalling in human platelets is mediated at least in part by PAR1 (refs 2–6, 12). A role for PAR3 in human platelets has not been demonstrated, but pharmacological studies have suggested that PAR1-independent mechanisms for activating human platelets may exist[3,17–20]. We have identified a human PAR4 cDNA (GenBank accession number AF080214) and studies using polymerase chain reaction (PCR) after reverse transcription of RNA suggest that it is expressed in human platelets (M.L.K., M. Nakanishi-Matsui and S.R.C., manuscript in preparation). In addition, both SFLLRN, a PAR1-activating peptide, and GYPGKF, a PAR4-activating peptide, activated human platelets; the mutant peptide GAPGKF did not (Fig. 4). The potency of these pharmacological tools for platelet activation correlated with their potency at their respective receptors in the oocyte system. GYPGKF activated human platelets at a threshold concentration of 300–500 μM and PAR4-expressing *Xenopus* oocytes with an EC_{50} (effector concentration for a half-maximal response) of 30 μM. SFLLRN activated human platelets at a threshold concentration of 1–3 μM and PAR1-expressing oocytes with an EC_{50} of 0.3 μM. SFLLRN showed no activity on PAR4-expressing oocytes and GYPGKF showed little activity on PAR1-expressing oocytes (<8% of maximal PAR1 activation even at 500 μM GYPGKF, data not shown). Lastly, GYPGKF activated PAR1-desensitized platelets (Fig. 4). These data suggest that PAR4 functions in human platelets.

In summary, PAR3 is necessary for normal thrombin signalling in mouse platelets but PAR4, a newly characterized thrombin receptor, also contributes. Human platelets also seem to use at least two

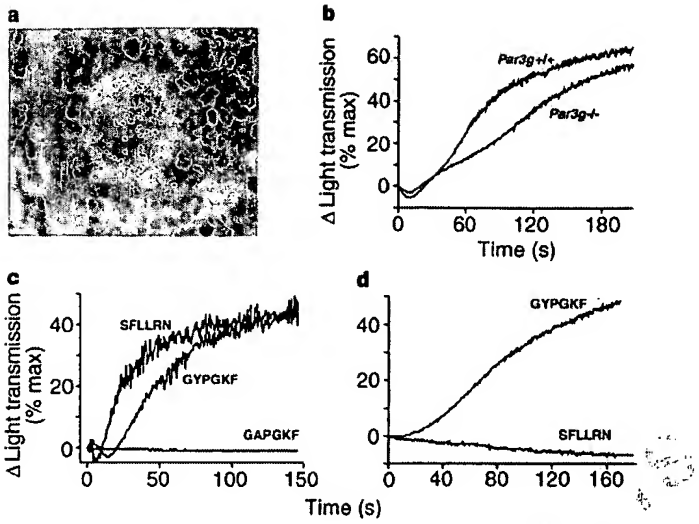

Figure 4 Expression of PAR4 in mouse megakaryocytes and evidence for PAR4 function in mouse and human platelets. **a**, *In situ* hybridization of mouse spleen for PAR4 mRNA. Bright-field photomicrograph shows silver grains overlying a megakaryocyte. Sense-probe controls were negative. **b**, GYPGKF, a PAR4-activating peptide, activates wild-type and PAR3-deficient mouse platelets. Stirred wild-type and PAR3-deficient platelets were exposed to GYPGKF (500 μM) at 0 s and aggregation was measured. Under the same conditions, no response to the control peptide GAPGKF was obtained (data not shown). This experiment was repeated three times. **c**, PAR4-activating peptide activates human platelets. Naive human platelets were exposed to the PAR1 agonist SFLLRN (3 μM), the PAR4 agonist GYPGKF (500 μM) or the mutant peptide GAPGKF (500 μM) and aggregation was measured. **d**, PAR4-activating peptide activates PAR1-desensitized human platelets. SFLLRN-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) and aggregation was measured. This experiment was replicated three times using platelets from two different donors. Note that GYPGKF activates naive and PAR1-desensitized human platelets.

receptors, PAR1 and PAR4, for thrombin signalling. Why might a two-receptor system for platelet activation by thrombin exist? It may simply provide redundancy in a pathway important for haemostasis. More interestingly, it may provide a mechanism for responding to proteases other than thrombin or to thrombin itself over a wider range of concentrations, for signalling with different tempos, for activating distinct downstream effectors, or for allowing differential regulation of receptor levels or function. The identification of this receptor system provides a framework for further defining the roles and relative importance of distinct PARs in platelets and other cells and for refining strategies for pharmaceutical development. For example, it may be necessary to block both PAR1 and PAR4 in human platelets to achieve an antithrombotic effect. Alternatively, the existence of a second receptor may provide a useful margin of safety for such potentially powerful therapeutic agents and/or the ability to block selected *in vivo* responses to thrombin.

Note added in proof: While this manuscript was in press, the identification of human PAR4 was reported independently[28]. □

Methods

Inactivation of the gene encoding PAR3 (*Par3g*). A bacterial artificial chromosome (BAC) that contained *Par3g* was obtained by PCR screen of a 129/SvJ mouse genomic library (Genome Systems). A 6.5-kilobase (kb) *Bam*H1/*Eco*RI fragment 5′ of exon 2 and a 2.0-kb *Bgl*II/*Bam*H1 fragment 3′ of exon 2 were cloned into the pNTK vector[21] to create the targeting vector (Fig. 1a). A 0.8-kb *Nde*I fragment of *Par3g* 3′ of the short arm of homology was used as a probe to identify both the wild-type and targeted alleles (Fig. 1a). RF8 ES cells[22] (129/SvJae) were electroporated with the targeting construct and clones that were resistant to G418 and FIAU were selected and screened by Southern blot. A highly chimaeric male mouse that was derived using $Par3g^{+/-}$ ES cells was bred to C57Bl/6 females to generate approximately 50 F_1 $Par3g^{+/-}$ mice. All experiments reported here were performed using the F_2 offspring of these mice.

Northern blot analysis. Poly(A)$^+$ RNA (3 μg) from wild-type and $Par3g^{-/-}$ mouse spleens was electrophoresed on a denaturing gel, transferred to reinforced nitrocellulose membrane (Schleicher & Schuell), and hybridized to a 600-base-pair (bp) *Eco*RI/*Sal*I PAR3 exon 2 probe under high stringency.

Flow cytometry. Washed mouse platelets were resuspended in platelet buffer (20 mM Tris-HCl pH 7.4, 140 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mg ml^{-1} glucose, 0.5% BSA, 1 μM PGE_1 and 5 mM EDTA), incubated with 10 μg ml^{-1} of anti-PAR3 IgG[23] at 4 °C for 1 h, washed, and then incubated with FITC-conjugated goat anti-rabbit IgG (Molecular Probes) at 4 μg ml^{-1} for 30 min. Platelets were then washed three times and analysed by flow cytometry.

Platelet aggregation and secretion. Blood was collected into citrate buffer from the inferior vena cava of pentobarbital-anaesthetized mice. Blood from 3–4 $Par3g^{-/-}$ mice or their wild-type littermates was pooled for each platelet study. Platelet-rich plasma was prepared by centrifugation of whole blood at 200 *g* for 7 min. EDTA (10 mM) and PGE_1 (1 μM) were then added and platelet-rich plasma was centrifuged at 500 *g* for 10 min. Platelets were then washed in platelet buffer containing 1 mM EDTA and 1 μM prostaglandin PGE_1, collected by centrifugation, resuspended to an OD_{500} of 1.0 (~2.5 × 10^8 platelets per millilitre) in platelet buffer lacking EDTA and PGE_1, and incubated on ice for 30 min before use. Calcium chloride was added to a final concentration of 2 mM and aggregation and secretion were measured in a Chrono-Log lumiaggregometer. Platelet suspension (300 μl) was added to the aggregometer chamber and change in light transmission after addition of agonist was followed. Results are expressed as the change (Δ) in light transmission, defined as the per cent increase in light transmission over that of the unactivated platelet suspension, with 100% representing light transmission of platelet buffer alone. Platelet ATP secretion was measured by adding luciferase (880 units per millilitre) and luciferin (8 μg ml^{-1}) to each sample; the luminescence generated by platelet-released ATP was compared with that of an ATP standard. Human blood was drawn from the antecubital vein; otherwise human platelets were prepared in a manner identical to mouse platelets. For PAR1-desensitization studies, human platelets that were resuspended from the first platelet pellet were incubated with 100 μM SFLLRN peptide at room temperature for 5 min without stirring, and then washed and resuspended as above.

Cloning of PAR4. Mouse EST 400689 was identified by BLAST search of the NCBI EST database using the coding region of human PAR2. The EST was sequenced and found to encode the carboxy-terminal 32 amino acids of a presumed G-protein-coupled receptor. A full-length cDNA was obtained by a combination of 5′ rapid amplification of cloned ends (RACE) using cDNA

from mouse embryo at day 14–15 (Marathon cDNA, Clonetech), and PCR of cDNA from a mouse brain endothelial cell line (bEND cells, W. Risau). A BAC mouse genomic clone was obtained from Genome Systems. The sequence of the cDNA (Fig. 3) and genomic clones were consistent. Human PAR4 was cloned from K562 mRNA by RT-PCR using primers based on mouse PAR4 sequence.

Functional studies in *Xenopus* oocytes. cDNA encoding Flag-epitope-tagged PAR4 (ref. 15) was constructed such that the Flag epitope was followed by amino-acid 18 of PAR4. Epitope-tagged and wild-type PAR4 cDNAs in pFROG[2] were transcribed *in vitro* and *Xenopus* oocytes were microinjected with 0.5–5.0 ng of PAR4 cRNA and 25 ng of PAR1 cRNA per oocyte. Agonist-triggered ^{45}Ca mobilization, a reflection of phosphoinositide hydrolysis in these cells, was measured[2,24].

***In situ* hybridization.** *In situ* hybridization[10] was done using sense or antisense ^{35}S-riboprobe transcribed from mouse PAR4 cDNA in pBluescript II SK. An 8-week exposure is shown.

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A mutation in succinate dehydrogenase cytochrome *b* causes oxidative stress and ageing in nematodes

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Much attention has focused on the aetiology of oxidative damage in cellular and organismal ageing[1–4]. Especially toxic are the reactive oxygen byproducts of respiration and other biological processes[5]. A *mev-1(kn1)* mutant of *Caenorhabditis elegans* has been found to be hypersensitive to raised oxygen concentrations[6,7]. Unlike the wild type, its lifespan decreases dramatically as oxygen concentrations are increased from 1 to 60% (ref. 7). Strains bearing this mutation accumulate markers of ageing (such as fluorescent materials and protein carbonyls) faster than the wild type[8,9]. We show here that *mev-1* encodes a subunit of the enzyme succinate dehydrogenase cytochrome *b*, which is a component of complex II of the mitochondrial electron transport chain. We found that the ability of complex II to catalyse electron transport from succinate to ubiquinone is compromised in *mev-1* animals. This may cause an indirect increase in superoxide levels, which in turn leads to oxygen hypersensitivity and premature ageing. Our results indicate that *mev-1* governs the rate of ageing by modulating the cellular response to oxidative stress.

Three-factor crosses using visible genetic markers placed *mev-1* between *unc-50(e306)* and *unc-49(e382)* on chromosome III (Fig. 1). We tested cosmids from this region for their ability to rescue *mev-1* mutants from oxygen hypersensitivity after germline transformation[10]. Cosmid T07C4, but not C38H2, M03C11 or other cosmids, was able to rescue the *mev-1* mutant phenotype (Fig. 1). By testing various subclones from this cosmid, we identified a 5.6-kilobase (kb) fragment that also restored wild-type resistance (Figs 1, 2). Rescue was essentially complete with respect to both oxygen hypersensitivity (Fig. 2a) and premature ageing (Fig. 2b). This fragment includes an open reading frame containing the conceptual gene *cyt-1*, which is homologous to the bovine succinate dehydrogenase (SDH) cytochrome b_{560} (ref. 11; GenBank accession number L26545 for *C. elegans* and S74803 for bovine, respectively). We found that the *mev-1(kn1)* strain contained a missense mutation resulting in a glycine-to-glutamic acid substitution in *cyt-1* (Fig. 3). This mutation created a restriction fragment-length polymorphism that enabled the restriction enzyme *Mro*I to cleave wild-type DNA but not *mev-1* DNA at position 323. As predicted from the sequences, digestion by *Mro*I of the products of polymerase chain reaction with reverse transcription (RT-PCR) yielded two bands from wild type (N2), one band from *mev-1(kn1)* and three bands from transgenic animals (*kn1;knIs2*) (Fig. 1). This confirms that the wild-type *cyt-1* gene that we introduced into the *mev-1* strain was expressed and that rescue was achieved by this gene. We also confirmed that a 2.8-kb wild-type PCR product of this gene, including regions from 1,566 bases upstream and 537 bases downstream, rescued *mev-1* (Fig. 1).